Universidade de Aveiro Secção Autónoma de Ciências da Saúde
2013

JULIANA CARINA CARDOSO FELGUEIRAS

O PAPEL DO COMPLEXO TCTEX1D4/PPP1 NA PROLIFERAÇÃO E MIGRAÇÃO CELULAR

THE ROLE OF TCTEX1D4/PPP1 COMPLEX IN CELL PROLIFERATION AND MIGRATION

Universidade de Aveiro Secção Autónoma de Ciências da Saúde
2013

JULIANA CARINA CARDOSO FELGUEIRAS

O PAPEL DO COMPLEXO TCTEX1D4/PPP1 NA PROLIFERAÇÃO E MIGRAÇÃO CELULAR

THE ROLE OF TCTEX1D4/PPP1 COMPLEX IN CELL PROLIFERATION AND MIGRATION

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar Convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro

Este trabalho é financiado por fundos FEDER através do Programa Operacional Fatores de Competitividade – COMPETE e por Fundos Nacionais através da FCT – Fundação para a Ciência e Tecnologia no âmbito do projeto “PTDC/QUI-BIQ/118492/2010” e pelo Centro de Biologia Celular.

o júri

presidente	Prof. Doutora Odete Abreu Beirão da Cruz e Silva Professora Auxiliar com Agregação da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro
orientador	Prof. Doutora Margarida Sâncio da Cruz Fardilha Professora Auxiliar Convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro
arguente	Prof. Doutora Carmen de Lurdes Fonseca Jerónimo Professora Associada Convidada com Agregação do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

agradecimentos

À Prof. Margarida Fardilha por ser uma orientadora presente e preocupada. Agradeço as oportunidades, a motivação, o apoio e o otimismo.

Ao Centro de Biologia Celular e à sua diretora, Prof. Doutora Odete da Cruz e Silva, por me terem acolhido e proporcionado as condições necessárias ao meu crescimento enquanto investigadora.

À FCT por ter suportado o desenvolvimento deste trabalho.

A todos os professores que me encaminharam durante a vida académica. Um agradecimento especial à Prof. Vera Afreixo por se ter disponibilizado, nesta última e determinante fase, a fazer-me olhar para a estatística de uma outra forma!

Aos meus companheiros e amigos do laboratório porque sem eles este ano de trabalho não teria sido tão excecional. Ao Korrodi por toda a ajuda, conhecimento e paciência, mesmo quando já tinha trabalho suficiente entre mãos. "*Porque se a vida fosse fácil...!"* À Joana e à Maria não só por toda a ajuda técnica e preocupação, mas também por me terem proporcionado ótimos momentos de descompressão que tanto contribuíram para levar este barco a bom porto. "*These are crazy days, but they make me shine.".* Ao Mega porque esteve lá em todos os momentos sem exceção e sem perder a paciência, o que nem sempre deve ter sido fácil! Sois verdadeiros Amigos*!*

Aos meus companheiros de curso que embarcaram comigo nesta aventura e que me deram a mão sempre que precisei. Não seria de todo justo não dar um lugar de destaque à Marta, que me vem aturando há 5 anos e com a qual partilhei todas as minhas alegrias e tristezas durante este tempo. Obrigada pela amizade incondicional! Mas não esquecendo também a Ana Maria, o Emanuel e a Patrícia, que sempre tiveram a palavra certa no momento indicado.

Ao Tiago pela ajuda preciosa, dedicação, e amizade. Obrigada por me ajudares a repor a paciência e o otimismo, com toda a calma que te é característica, mesmo quando eu achava que já não era possível.

Aos colegas do Laboratório de Neurociências e do Laboratório de Biogénese de Organelos na Saúde e na Doença pela ajuda e momentos de companheirismo e cumplicidade.

E, acima de tudo, à minha família, especialmente aos meus pais e à minha irmã. Porque a eles devo grande parte do que hoje sou. Deram sempre o melhor deles e incentivaram-me desde cedo a dar o melhor de mim. *"Sê todo em cada coisa. Põe quanto és no mínimo que fazes"*. Agradeço o suporte, a confiança, o amor e o carinho.

A todos os que, de uma forma ou de outra, contribuíram para este trabalho.

palavras-chave

TCTEX1D4, fosfoproteína fosfatase 1 (PPP1), proteínas interactoras da PPP1 (PIPs), fosforilação reversível, vias de sinalização, TGFβ, cancro da próstata, proliferação celular, migração celular.

resumo

O cancro da próstata é um dos cancros mais prevalentes e incidentes em homens, sendo uma preocupante causa de morte a nível mundial. A ampla utilização do antigénio específico da próstata (PSA) no seu rastreio e as melhorias na sua terapêutica têm permitido uma diminuição marcada da mortalidade. No entanto, o uso do PSA é controverso uma vez que se verifica um diagnóstico excessivo e o tratamento de vários casos que, eventualmente, não representariam qualquer ameaça para a vida do individuo. Assim, o estabelecimento de novos biomarcadores e a descoberta de novos alvos terapêuticos para o cancro da próstata constitui um importante desafio na atualidade.

Alterações em várias vias de sinalização têm sido relacionadas com o desenvolvimento e a progressão do cancro da próstata. A via do TGFβ é uma das vias mais proeminentes no controlo do crescimento da próstata, uma vez que contrabalança os efeitos proliferativos dos androgénios. Um dos principais mecanismos de regulação da via do TGFβ é a fosforilação reversível, executada de modo complementar por proteínas cinases e fosfatases. Uma das fosfatases envolvidas na regulação da via do TGFβ é a PPP1.

A PPP1 é uma fosfatase reguladora de inúmeros eventos celulares. A maioria das suas funções envolve a sua ligação a subunidades reguladoras conhecidas como PIPs. Recentemente, foi descrita uma nova PIP, a TCTEX1D4, que já havia sido referida como interactor dos recetores da via do TGFβ. No entanto, pouco se sabe acerca da sua função.

Este trabalho permitiu identificar a TCTEX1D4 como agente inibidor da proliferação em células humanas de próstata. A sua atividade parece ser modulada através da ligação à PPP1, uma vez que a disrupção do complexo TCTEX1D4/PPP1 conduziu a uma maior inibição da proliferação celular. Por outro lado, a TCTEX1D4 pode regular indiretamente a atividade da PPP1 ao modular a sua localização no interior da célula. Este complexo não parece atuar ao nível da migração celular.

Tendo em conta os presentes resultados, o complexo TCTEX1D4/PPP1 parece constituir um potencial alvo para o desenvolvimento de novas terapias para o cancro da próstata.

keywords

t-complex testis expressed 1 domain containing 4 (TCTEX1D4), phosphoprotein phosphatase 1 (PPP1), PPP1-interacting proteins (PIPs), reversible phosphorylation, cell signaling pathways, transforming growth factor-β (TGFβ), prostate cancer, cell proliferation, cell migration.

abstract

Prostate cancer is one of the most prevalent and incident cancer in men, being a disquieting cause of men's death worldwide. The widespread implementation of prostate-specific antigen (PSA) screening and the improvements on prostate cancer therapy have been leading to a markedly decrease in mortality. However, PSA use is controversial since it has been leading to the diagnosis and treatment of several prostate cancer cases that would not otherwise cause symptoms or threaten man's life. Hence, the establishment of a panel of biomarkers and the discovery of new therapeutical targets for prostate cancer is a pressing need.

Changes in several cell signaling pathways have been associated with de onset, development, and progression of prostate cancer. TGFβ signaling counterbalanced the mitogenic effects of androgens, thus being one of the most prominent pathways involved in controlling prostate growth. Mediators of TGFβ signaling pathway are strictly regulated by several mechanisms, among which reversible phosphorylation. This process involves a fine equilibrium between the action of protein kinases and phosphatases.

PPP1 is a major serine/threonine phosphatase that regulates numerous cellular events, including TGFβ signaling. The majority of its functions are accomplished by association with regulatory subunits, known as PPP1-interacting proteins (PIPs).

Recently, a novel PIP was found: TCTEX1D4, a dynein light chain protein that was already established as an interactor of TGFβ receptors. Its functions, nevertheless, remain poorly understood.

This work identified TCTEX1D4 as an anti-proliferative agent in human prostate cells. Also, TCTEX1D4 activity seems to be regulated through binding to PPP1, as the disruption of TCTEX1D4/PPP1 complex resulted in enhancement of cell proliferation inhibition. Alternatively or in addition, TCTEX1D4 may indirectly regulate PPP1 function by modulating its subcellular localization. This complex doesn´t appear to play a major role in cell migration.

Given the results, TCTEX1D4/PPP1 complex may constitute a potential target for the development of new prostate cancer therapies.

TABLE OF CONTENTS

FIGURES

TABLES

ABBREVIATIONS

AB	alamarBlue
Abl	Abelson tyrosine-protein kinase
ALK	activin receptor-like kinase
AMACR	alpha-methylacyl-CoA racemase
AMH	anti-müllerian hormone
APS	amonium persulfate
AR	androgen receptor
ASAP	atypical small acinar proliferation
ATP	adenosine-5'-triphosphate
BCA	bicinchoninic acid
BMPs	bone morphogenetic protein
BPE	bovine pituitary extract
BPH	benign prostate hyperplasia
BRCA2	breast cancer 2, early onset
BSA	bovine serum albumin
CD	cluster of differentiation
Cdc42	cell division control protein 42 homolog
cDNA	complementary DNA
CHEK2	checkpoint kinase 2
Co-Smad	common Smad
CTD	carboxy-terminal domain
DAPI	4',6-Diamidino-2-Phenylindole
DHC	dynein heavy chain
DHT	dihydrotestosterone
DIC	dynein intermediate chain
DLC	dynein light chain
DLIC	dynein light intermediate chain
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

E-cadherin	epithelial cadherin
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
ENG	endoglin
ERK	extracellular signal-regulated kinase
EST	E-twenty six
EZH2	enhancer of zeste homolog 2
FAK	focal adhesion kinase
FBS	fetal bovine serum
FCP	TFIIF-associating CTD phosphatase
GADD34	growth arrest and DNA damage-inducible protein 34
GDFs	growth differentiation factors
GST-π	glutathione-S-transferase π
GTP	guanosine-5'-triphosphate
HDACs	Histone deacetylases
HepG2	hepatocellular carcinoma cells
HMEC-1	human dermal microvascular endothelial cell line
HPC	hereditary prostate cancer
HUVECs	as human umbilical vein endothelial cells
I-Smad	inhibitory Smad
JNK	stress-activated protein kinase
K-SFM	keratinocyte serum free medium
LAP	latency-associated peptide
LCLC	large cell lung cancer
LGB	lower gel buffer
MAPK	mitogen-activated protein kinase
miRNA	microRNA
MSR1	macrophage scavenger receptor 1 gene
NFκB	nuclear factor κB
NIPP1	nuclear inhibitor of protein phosphatase 1
P	phosphate
PBS	phosphate buffered saline

PCa	prostate cancer
PCR	polymerase chain reaction
PDK1	pyruvate dehydrogenase lipoamide kinase isozyme 1
PECAM-1	platelet-endothelial cell adhesion molecule 1
PIA	proliferative inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PIP	PPP1-interacting protein
PKB	protein kinase B
PP	protein phosphatase
PPM	protein phosphatases Mg^{2+}- or Mn^{2+}-dependent
PPP	phosphoprotein phosphatase
PPP1	phosphoprotein phosphatase 1
PPP1BM	PPP1 binding motif
PPP1C	PPP1 catalytic subunit
PPP1CA	PPP1 isoform alpha
PPP1CB	PPP1 isoform beta
PPP1CC	PPP1 isoform gamma
pRB	retinoblastoma protein
PSA	prostate-specific antigen
PTEN	phosphatase and tensin homologue
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
Rac1	Ras-related C3 botulinum toxin substrate 1
Rb	retinoblastoma tumor suppressor gene
RhoA	Ras homolog gene family, member A
RNA	ribonucleic acid
RNASEL	ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)
RPMI	Roswell park memorial institute medium
R-Smad	receptor-regulated Smad
RT	room temperature
SARA	Smad anchor for receptor activation
SCP	small CTD phosphatase

SD	Standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
STAT3	signal transducer and activator of transcription 3
STPP	serine/threonine protein phosphatase
SV40	simian vacuolating virus 40
TBS	tris buffered saline
TBST	tris buffered saline with tween
TCTEX1D4	t-complex testis expressed 1 domain containing 4
TE	transfection efficiency
TF	transcription factor
TGFβ	transforming growth factor-β
TMPRSS2	transmembrane protease serine 2
TβRI	type I TGFβ receptor
TβRII	type II TGFβ receptor
TβRIII	type III TGFβ receptor
UGB	upper gel buffer
VCAM-1	vascular cell adhesion molecule 1
WR	working reagent

INTRODUCTION

1.1 OVERVIEW OF THE HUMAN PROSTATE AND PROSTATE CANCER

1.1.1 ANATOMY AND PHYSIOLOGY OF THE PROSTATE GLAND

The prostate is an accessory gland of the male reproductive system. As depicted in Figure 1A, it is located in the pelvis, under the bladder, and surrounding the prostatic urethra [1].

As an exocrine gland, the prostate produces a milky secretion, known as prostatic fluid, essential for the formation of the seminal fluid (it represents near 30% of the seminal fluid volume). The prostatic fluid maintains the fluidity of the seminal fluid, nourishes the sperm, and supports the fertilization process [1]. Moreover, the prostate supports the control of urine output and ejaculation, and participates in the metabolism of testosterone [2].

The prostate is constituted by glandular and non-glandular structures. Among the glandular tissue, four anatomical zones can be distinguished: (1) the peripheral zone (70% of the glandular tissue); (2) the central zone (25% of the glandular tissue); (3) the transition zone (5% of the glandular tissue); and (4) the periurethral zone, which encompasses mucosal and submucosal glands (Figure 1B) [3]. Prostate glandular structures are embedded in a fibromuscular stroma (Figure 1B) [4].

These zones present different embryologic origins, histology, function and susceptibility to pathological processes is also distinct [5].

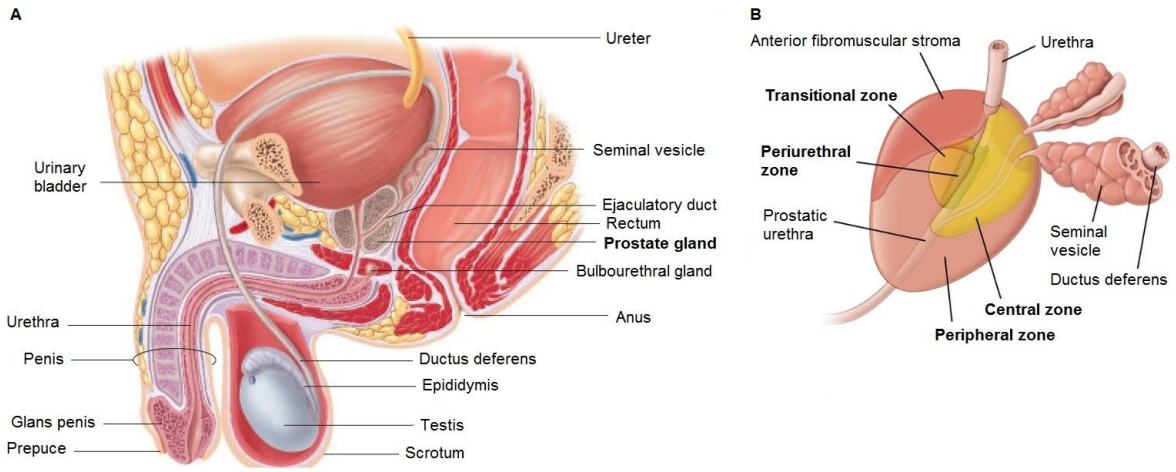


Figure 1 | Overview of the human prostate gland localization and anatomy.

(A) Localization of the human prostate in a sagittal section of the pelvic cavity. (B) Anatomical zones of the human prostate gland. [Adapted from [6, 7]].

In terms of cellular content, the prostate epithelium contains three main cell types which exhibit distinct expression profiles. Secretory luminal cells mostly express androgen receptor (AR), cytokeratins 8 and 18, and cluster of differentiation 57 (CD57). They are responsible for the production of prostate-specific antigen (PSA), prostatic acid phosphatase, and human kallikrein-2. Basal cells express cytokeratins 5 and 14, CD44, and other markers at a less extent. They also express low levels of AR and are believed to be the proliferative compartment of the prostate. Neuroendocrine cells are androgen-independent and express chromogranin A and a variety of peptide hormones [8, 9].

The growth of the glandular epithelium is influenced by sexual hormones, with emphasis to testosterone and dihydrotestosterone (DHT), the most important mitogens for the normal prostate. In the classical AR signaling, ligands bind to the AR in the cytosol resulting in its translocation into the nucleus where it acts as a transcription factor. AR binds to specific sequences of deoxyribonucleic acid (DNA) in the promoter regions of target-genes, known as androgen-response elements, and activates genes that, overall, stimulate proliferation, differentiation, and secretion [3, 10]. On the other hand, androgens potentiate the activation of alternative signaling molecules, including epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor-α (TGFα), and insulin-like growth factor

(IGF). Proliferative effects of such molecules are counterbalanced by transforming growth factor-β (TGFβ).

1.1.2 PROSTATE CANCER EPIDEMIOLOGY AND PATHOGENESIS

The prostate tissue is mentioned as the original site for the two most prevalent diseases of elderly men: benign prostatic hyperplasia (BPH) and prostate cancer (PCa) [5]. PCa was considered by the Globocan Project 2008 the second most incident cancer in men (only excelled by lung cancer), with nearly 900 000 men being affected each year worldwide. It was also identified as the sixth leading cause of death from cancer among men worldwide. In Portugal, PCa is the most incident and prevalent cancer in men and represents the third cause of cancer-related death [11, 12].

Old age, black ethnicity, and a family history of the disease are the risk factors most commonly associated with PCa. The average age at the time of diagnosis is 67 and about two-thirds of cases are diagnosed in men aged 65 years and over–diagnosis before age 40 is rare. Environmental risk factors such as eating habits, early sexual initiation, and sexually transmitted infections are also associated with the development of the disease [13].

About 70% of the PCa cases originate in the peripheral zone, 15%-20% in the central zone, and only 10%-15% in the transition zone (in contrast to the BPH that mainly develops in the transition zone) [14].

The cellular origin of PCa has been attributed either to the dedifferentiation and mortality acquisition of differentiated luminal cells or to the malignant transformation of prostate stem cells that are confined to the basal compartment [15]. These prostate stem cells consist of a slowly proliferating population that gives rise to a population of progenitor cells, called transient amplifying population, which in turns generate the mature prostatic epithelial cells in response to androgens [8]. Although these cells are androgen-sensitive in terms of growth, they do not rely on androgens for survival [5]. It has also been observed that PCa can arise from basal cells, although the aggressive potential of luminal and basal cells populations differs [16].

Similarly to other cancer types, precancerous lesions seem to occur prior to PCa development (Figure 2). Overall, loss of tumor suppressor genes, oncogene activation, and epigenetic alterations culminate in the activation of growth factor receptors, signaling

mediators, kinases, transcription factors, coregulators, and multiple proteases that enable the progression to PCa (Figure 2) [17, 18].

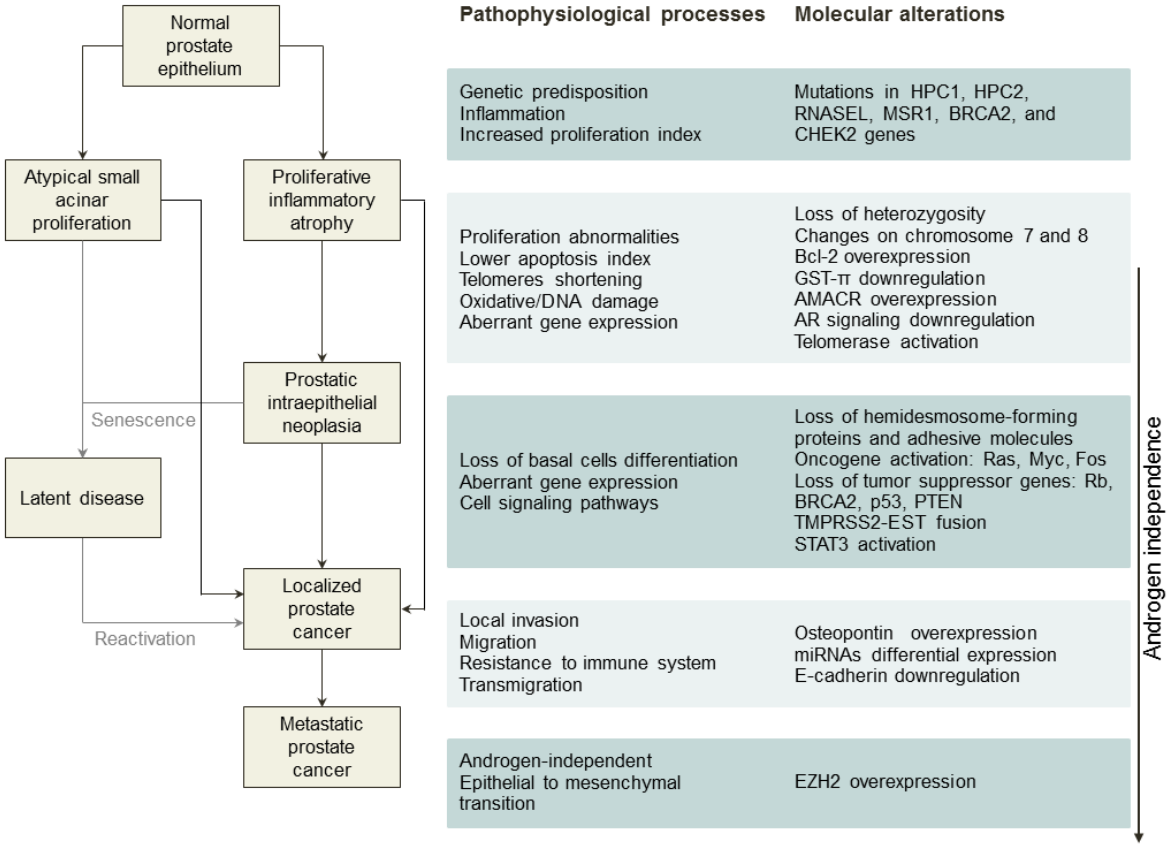


Figure 2 | Stages and processes underlying PCa initiation and development.

The scheme on the left represents the stages of PCa progression leading to a metastatic and androgen-independent stage. On the right, each blue rectangle corresponds to the progression phase on the left. AMACR, alpha-methylacyl-CoA racemase; BRCA2, breast cancer 2, early onset; CHEK2, checkpoint kinase 2; EST, E-twenty six; EZH2, enhancer of zeste homolog 2; GST-π, glutathione-S-transferase π; HPC1, hereditary prostate cancer 1; HPC2, hereditary prostate cancer 2; miRNAs, microRNAs; MSR1, macrophage scavenger receptor 1 gene; p53, protein 53; PTEN, phosphatase and tensin homologue; Rb, retinoblastoma tumor suppressor gene; RNASEL, ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent); STAT3, signal transducer and activator of transcription 3; TMPRSS2, transmembrane protease serine 2. [Adapted from [18]].

The major problem arising from PCa is its propensity to metastasize. A large number of specific molecular mechanisms lead to local invasion, extravasation and distal migration from the primary site and subsequently to endothelial attachment, transmigration and site-specific establishment of metastases at secondary sites, usually at the bone, lung or liver (Figure 2) [19, 20]. Of extreme importance is the down-regulation of cell-cell and cell-matrix

characteristics, which enable malignant cells to become motile, and their ability to destroy the extracellular matrix through degradative enzymes [20].

1.2 TGFβ SIGNALING IN PROSTATE CANCER

1.2.1 TGFβ SIGNALING PATHWAY OVERVIEW

TGFβ is a multifunctional cytokine that belongs to the TGFβ superfamily, which also includes bone morphogenetic proteins (BMPs), activins/inhibins, growth differentiation factors (GDFs), and the anti-müllerian hormone (AMH), among others. TGFβ superfamily members have been implicated in numerous behavioral aspects of cells, including proliferation, differentiation, apoptosis, and motility [21-24].

The adult human prostate mostly expresses three TGFβ isoforms: TGFβ1, the most abundant and ubiquitously expressed isoform, TGFβ2, and TGFβ3 [24, 25].

TGFβ ligands are synthesized as latent pre-pro-peptides with high molecular weight that encompass a longer N-terminal pro-peptide (latency-associated peptide, LAP) followed by a shorter C-terminal mature polypeptide. During the secretory pathway, the pro-peptide is proteolytically cleaved from the mature peptide in the trans-Golgi apparatus by furin-like proteases, but they remain noncovalently linked [24]. This complex might also associate with latent TGFβ binding proteins, forming a large latent complex, which is important for the regulation of TGFβ bioavailability. In the extracellular matrix, via further proteolytic cleavage and/or structural modification of the complex, TGFβ is converted to a dimeric active cytokine that is able to bind with high-affinity to cell surface receptors [23, 24, 26].

TGFβ signals mainly through interaction with the extracellular domain of two types of transmembrane serine/threonine kinase receptors: the type I TGFβ receptor (TβRI, also known as activin receptor-like kinase, ALK) and the type II TGFβ receptor (TβRII). In humans, seven TβRI and five TβRII are currently characterized. All of them are constituted by three regions: (1) an extracellular N-terminal domain responsible for TGFβ binding, (2) a transmembrane domain; and (3) a cytoplasmic C-terminal domain which incorporates the catalytic center. Unlike TβRII, which are constitutively active, TβRI requires phosphorylation of the GS domain (a serine and threonine rich region) to become active. Therefore, ligands bind specifically to TβRII that, in turn, phosphorylates TβRI in the

cytosolic GS domain and thereby activates it. Afterwards, the active heterotetrameric receptor complex activates downstream signaling pathways [23, 24].

TGFβ signaling is modulated by coreceptors that lack the kinase domain, as betaglycan (also referred as type III TGFβ receptor, TβRIII) and endoglin (ENG) [24]. Both receptors are on the cytoplasmic membrane but can also be found in a soluble form [23]. TβRIII, the largest and most abundant, binds all TGFβ isoforms; on the other hand, ENG primarily binds TGFβ1 and TGFβ3 [24].

As observed in other signaling pathways, TGFβ can activate two intracellular pathways: the canonical and the non-canonical (Figure 3).

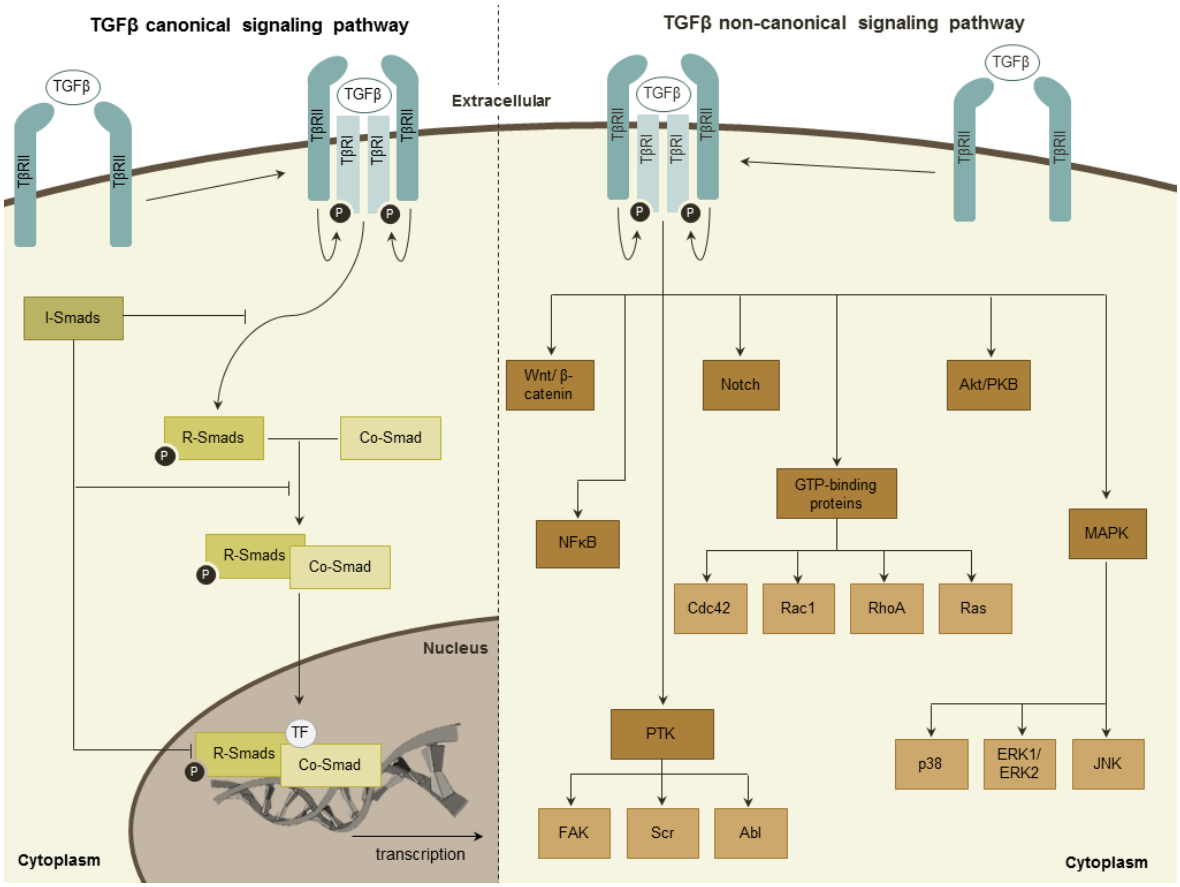


Figure 3 | TGFβ canonical and non-canonical signaling pathway.

In the canonical signaling pathway (left), R-Smads are activated by serine phosphorylation and form a complex with Co-Smad. The complex is translocated into the nucleus where it can bind to specific transcription factors (TF) and induces the transcription of TGFβ-dependent genes. On the other hand, TGFβ can activate alternative signaling pathways (right). Abl, Abelson tyrosine-protein kinase; Cdc42, cell division control protein 42 homolog; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; JNK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; P, phosphate; PKB, protein kinase B; PTK, protein tyrosine kinase; Rac1, Ras-related C3 botulinum toxin substrate 1; RhoA, Ras homolog gene family, member A.

Canonical signaling pathway effectors are classified as receptor-regulated Smads (R-Smads), common Smad (Co-Smad) and inhibitory Smads (I-Smads). R-Smads include Smads 1/2/3/5/8 and are direct targets of TβRI, propagating signals towards the nucleus. Co-Smad, or Smad4, forms heterotrimeric complexes with R-Smads and aids the propagation of the signal. Signal is transmitted in either two ways: (1) via Smad2/3, which are phosphorylated by the TβRI ALK4/5/7, mainly activated by TGFβ, nodal, activins, and some GDFs; or, (2) via Smad1/5/8, which are phosphorylated by the TβRI ALK1/2/3/6, mostly activated by BMPs, AMH, and some GDFs. Upon formation of the heterotrimeric complex, it is translocated to the nucleus via microtubules and dyneins, where it modulates the expression of several genes (Sp1, Id1, Id2, and Myc). I-Smads, Smads 6 and 7, antagonize the TGFβ signaling: (1) through receptor binding, avoiding R-Smads/receptor interaction and signal propagation; (2) by preventing the formation of R-Smad/Co-Smad complexes; (3) by targeting receptors to degradation; or (4) through binding to DNA and Smad complexes in the nucleus, blocking the signal [21, 23].

TGFβ can also activate non-canonical pathways, and thus participate in cell proliferation, differentiation, migration, apoptosis, epithelial to mesenchymal transition, and matrix formation [24].

1.2.2 REGULATION OF THE TGFβ SIGNALING PATHWAY

TGFβ signaling is tightly regulated due to the TGFβ critical role on normal cell behavior and its involvement in pathological conditions. This regulation is observed at diverse levels of the pathway, involving ligands, receptors, and Smads. As mentioned above, TGFβ ligands are synthesized as a precursor protein that contains a LAP able to maintain the ligands in a latent form; ligands access to the kinase receptors is modulated by coreceptors; and I-Smads inhibit the signaling in different ways [27].

Furthermore, the internalization and recycling of ligand-receptor complex are important steps in the regulation of the signal [27]. Activated receptor endocytosis occurs in early endosomes and involves the Smad anchor for receptor activation (SARA) in cooperation with the regulatory adaptor cytoplasmic promyelocytic leukemia tumor suppressor protein. SARA facilitates the phosphorylation and activation of the TβRI through recruitment of Smad2/3. Alternatively, TGFβ receptors degradation can occur via caveolae, characterized by the presence of caveolin-1 and key regulatory proteins, such as Smad7 and Smurf [26].

Additional TGFβ signaling regulators are motor proteins that are associated with microtubules. For instance, R-Smads, either before or after phosphorylation, can interact with kinesin and dynein light chain proteins to accomplish their trafficking.

TGFβ receptors and Smads are also controlled by a number of post-translational modifications, such as reversible phosphorylation, ubiquitination, SUMOylation and acetylation [28]. TGFβ pathway is activated by a sequence of phosphorylation reactions; therefore, dephosphorylation of TGFβ signaling intervenients constitutes a counterbalance mechanism that limits the duration and intensity of the signal, contributing to its termination [29].

1.2.3 IMPLICATIONS OF THE TGFβ SIGNALING IN HUMAN PROSTATE CANCER

TGFβ has a dichotomous function in human cancers. In spite of its physiological inhibitory effects and its role as tumor suppressor in early tumorigenesis, it also acts as a tumor promoter in later stages of tumor progression [18, 30]. However, the precise mechanism underlying this phenomenon remains poorly defined.

TGFβ has been shown to directly or indirectly mediate several cancer hallmarks, such as self-sufficiency in growth factors, insensitivity to anti-growth signals, loss of apoptotic potential, limitless replicative potential, evasion of the immune system, tissue invasion and metastasis formation, and sustained angiogenesis [25]. The exact cellular effect, nonetheless, depends on the cell type and physiological conditions [24].

As observed in other types of cancer, PCa cells become resistant to the inhibitory effects of TGFβ, while maintaining some TGFβ-mediated responses, such as the stimulation of motility [20].

PCa cells express high levels of TGFβ1. These increased levels aid tumorigenesis by acting directly on the tumor cells themselves and indirectly by stimulating angiogenesis and suppressing the immune system [31, 32]. Thus, TGFβ1 levels correlate with high Gleason Score, bone metastasis formation, angiogenesis and poor clinical outcome [5].

Regarding TGFβ receptors, there is a decrease in both TβRI and TβRII [33]. Actually, circa 30% of PCa specimens exhibit downregulation or absence of a TGFβ receptor [20]. The absence of these receptors in PCa cells leads to growth inhibition resistance, enabling a clonal expansion of these cells [33-35].

Several studies have also reported a loss of TβRIII, mainly during metastasis formation. This loss is associated with disease state, metastatic disease, and PSA recurrence [36]. Remarkably, other studies demonstrated that ENG suppresses human PCa metastasis. Indeed, ENG expression facilitates ALK2-mediated Smad1 activation, thereby suppressing TGFβ-mediated increases in PCa cell motility. Contrastingly, with PCa progression, the decrease of ENG expression contributes to the predomination of ALK5-Smad3 pathway, thus promoting cell motility [37].

The activity of Smad intracellular signaling in PCa has also been lengthily explored. In the earlier stages of PCa development, ALK2-Smad1/5/8 signaling prevails to increase the growth and neovascularization. On the other hand, in advanced PCa this signaling pathway is overcame by ALK5-Smad2/3 signaling, leading to the enhancement of invasiveness, migration and metastasis formation [38, 39].

The increasing evidence of the relevance of TGFβ signaling in PCa carcinogenesis has led to the challenging idea that the target of its mediators should provide a mechanism of controlling tumor progression [40]. Indeed, preclinical studies using antisense approaches and antibodies to target TGFβ mechanisms, as well as the use of small molecule inhibitors that act through TGFβ receptors have been quite promising [41]. Also, genistein, that was found to act through Smad1 in an ALK2-dependent way to suppress PCa cell invasion, is already ongoing phase II clinical trials [42].

1.3 PHOSPHOPROTEIN PHOSPHATASES

1.3.1 OVERVIEW ON REVERSIBLE PROTEIN PHOSPHORYLATION

Reversible protein phosphorylation is the most common post-translational modification controlling intracellular metabolic mechanisms in eukaryotic cells. Phosphorylation of a protein may induce conformational changes in the protein, create binding sites for effector molecules, activate or inhibit enzymes, alter the association/dissociation properties of protein complexes, stabilize proteins, target proteins to degradation, and promote/inhibit the movement of subcellular components [43]. Unsurprisingly, deregulation of such regulatory system underlies many pathological states (e.g. cancer, diabetes and neurodegenerative conditions) [44].

Phosphorylation corresponds to the addition of a phosphate group to an amino acid side chain of a protein by a protein kinase; the phosphate group is normally provided by an adenosine-5'-triphosphate (ATP) molecule and the most common targets are the hydroxyl groups of serine, threonine, or tyrosine. Dephosphorylation is the opposite reaction, in which a phosphatase catalyzes the removal of the phosphate group from the phosphoprotein [45].

The first reference to this process remounts to the late 20s, when Cori and Cori started their investigation about glycogenolysis and glycogenesis (actually known as Cori cycle). The enzymatic intervenients in these reactions were later defined by Edmond Fischer and Edwin Kreb as being a protein kinase (phosphorylase *a*; phosphorylase kinase) and a protein phosphatase (phosphorylase *b*; phosphorylase phosphatase) [45, 46]. Therefore, reversible protein phosphorylation reactions are catalyzed by kinases and phosphatases in a counterbalanced way [44].

In human genome, nearly 3% of all eukaryotic genes encode protein kinases or, at less extent, protein phosphatases; there are only 150 phosphatases comparing to the near 500 protein kinases [44, 47].

According to the substrate specificity, protein phosphatases are divided into two main groups: serine/threonine protein phosphatases (STPPs) and protein tyrosine phosphatases (PTPs) [45]. Each group includes several families and subfamilies (Figure 4).

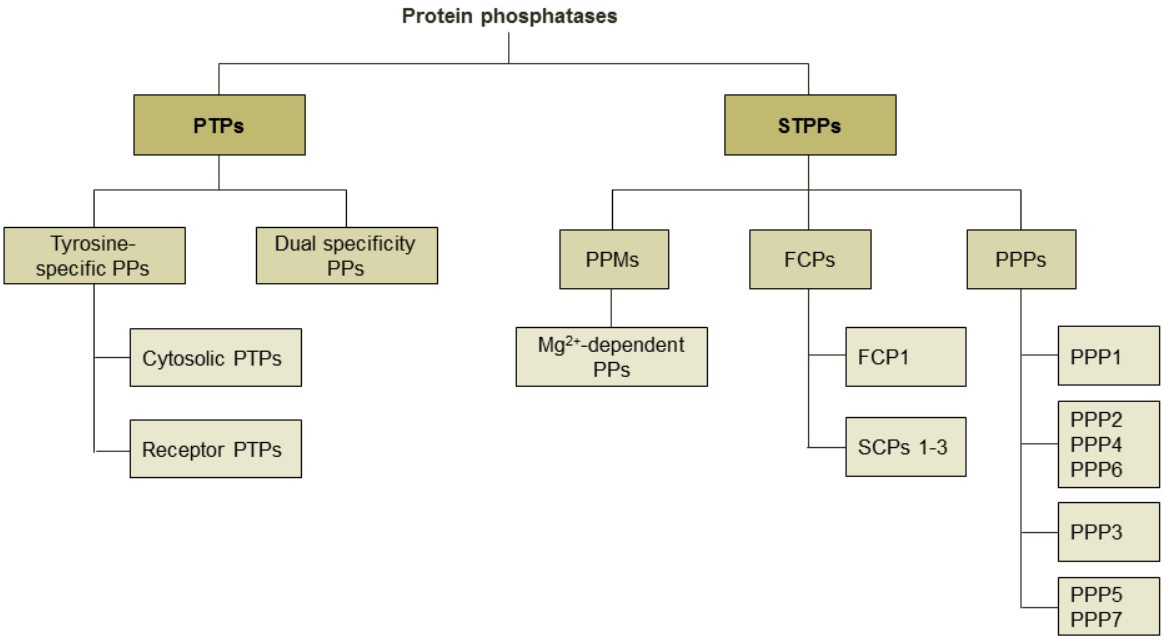


Figure 4 | Families of protein phosphatases (PPs).

Protein phosphatases are divided into two major groups: PTPs and STPPs. PTPs include the tyrosine-specific PPs, which can be either cytosolic or receptor PTPs, and the dual-specificity phosphatases, capable of dephosphorylating serine, threonine, and tyrosine residues. STPPs comprise three gene families: protein phosphatases Mg^{2+}- or Mn^{2+}-dependent (PPMs), TFIIF-associating C-terminal domain (CTD) phosphatases (FCPs), and phosphoprotein phosphatases (PPPs). PPMs include Mg^{2+}-dependent PPs, such as pyruvate dehydrogenase and PPM1. FCPs include FCP1 as well as small CTD phosphatases (SCPs). PPPs family encompasses PPP1-7.

1.3.2 PPP1 AND ITS INTERACTING PROTEINS ON PROSTATE CANCER

Phosphoprotein phosphatase 1 (PPP1) is a major STPP involved in a wide range of cellular processes, including cell division, apoptosis, protein synthesis, metabolism, cytoskeletal reorganization, and regulation of membrane receptors and channels [45, 48, 49].

PPP1 holoenzymes exhibit a highly conserved catalytic subunit (PPP1C). In humans, PPP1C is encoded by three genes, resulting in the isoforms PPP1CA, PPP1CB and PPP1CC (which can form PPP1CC1 and PPP1CC2 by tissue-specific alternative splicing). With exception of PPP1CC2, which is testis-enriched and sperm-specific, PPP1 isoforms are ubiquitously expressed although their expression levels and subcellular localization vary within different cell types [47, 48].

PPP1C binds to a number of regulatory subunits, known as PPP1 interacting proteins (PIPs). PIPs allow the versatility of PPP1 by modulating its subcellular localization and substrate affinity. Almost all PIPs share a short and degenerated RVxF motif, a primary

docking site that binds to a hydrophobic groove on a surface behind the PPP1C active site [44].

Several PIPs have been identified and actually more than two hundred PIPs are recognized as modulators of PPP1 subcellular localization and activity [44, 48-50]. Therefore, increasing attention has been given to the identification and characterization of PIPs not only to establish PPP1 functions but also to understand its role in pathologic conditions. For instance, PPP1-PIP complexes were shown to regulate two major tumor suppressors, p53 and retinoblastoma protein (pRb) [51, 52].

PPP1/PIP complexes have been identified as intervenients on PCa. Fer is a tyrosine kinase whose levels are significantly higher in PCa [53]. Upregulation of Fer in PCa cells leads to the inactivation of PPP1, resulting in the hyperphosphorylation of pRB and, thus, carcinogenesis promotion [54, 55].

Caveolin-1, an integral membrane protein, is overexpressed in PCa cells and is associated with the disease progression [56]. It was shown to inhibit PPP1, leading to the increase of pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1), PKB, and ERK1/2 activities. Consequently, caveolin-1 is able to maintain Akt signaling activated and sustain the activation of downstream oncogenic Akt targets, increasing the cell survival [57].

Chen and colleagues observed the interaction between PPP1 and AR: (1) PPP1 inhibition enhanced proteasome-mediated AR degradation; (2) PPP1 overexpression increased AR expression and markedly enhanced AR transcriptional activity in PCa cells. PPP1 regulates AR stability and nuclear localization through dephosphorylation of Ser-650. Thence, AR may function as a PPP1 regulatory subunit, mediating PPP1 recruitment to chromatin, where it can modulate transcription and splicing [58].

Recently, PPP1/nuclear inhibitor of protein phosphatase 1 (NIPP1) complex was described as a regulator of PCa cells directional migration [59].

As imbalances in protein phosphorylation system underlie numerous pathogenic processes, modulation of kinases and phosphatases activity might be an attractive therapeutical target [18]. The targeting of kinases has been widely explored, but efforts are now being made by pharmaceutical companies to also investigate the role of phosphatases. However, targeting a specific phosphatase has been associated with severe secondary effects, as nephrotoxicity and hepatotoxicity. For this reason, the adequate option seems to be targeting PIPs instead, as they are more event, tissue and subcellular compartment specific [44]. Two targeted

PPP1/PIP complexes have already been described. The PIPs involved are the growth arrest and DNA damage-inducible protein 34 (GADD34) and histone deacetylases (HDACs). PPP1/GADD34 complex is diminished in cells treated with salubrinal – a small molecule that protect the cell from ER-stress-induced apoptosis; and trichostatin A disrupts PPP1/HDAC6 complex in glioblastoma and PCa cells [60]. Hence, to unravel the complete PPP1 interactome in PCa is of crucial importance to establish potential new therapeutical targets.

1.3.3 TGFβ SIGNALING PATHWAY REGULATION BY PPP1

Despite the longstanding suspected influence of protein phosphatases in the regulation of TGFβ signaling, concrete data only started to emerge recently.

PPP1-PIP complexes have been implicated in the TGFβ signaling. By being targeted through the PPP1-docking protein SARA, PPP1C dephosphorylates TβRI, resulting in the attenuation of the signal [61]. This targeting also involves Smad7 and GADD34 [62]. Moreover, Smad7 may recruit PPP1C to ALK1 thereby mediating its dephosphorylation and inactivation in endothelial cells [63].

Recently, a novel PIP has been identified using a yeast two-hybrid screen from a human testis complementary DNA (cDNA) library: t-complex testis expressed protein 1 domain containing 4 (TCTEX1D4), a dynein light chain family member that has been shown to have a role in the TGFβ cascade [64].

1.4 TCTEX1D4, A NOVEL PPP1-INTERACTING PROTEIN

1.4.1 OVERVIEW ON DYNEINS CHARACTERIZATION

Dyneins are multimeric molecular motor complexes that translocate cellular cargo along microtubules, minus-end directed. They are composed of four subunits: dynein heavy chains (DHCs), intermediate chains (DICs), light intermediate chains (DLICs), and light chains (DLCs) (Figure 5). DHCs, which exhibit both ATPase and microtubule motor activities, associate with DICs and DLCs to form the basal cargo-binding domain of the enzyme [65, 66]. DLCs bind to the cargo and determine the cargo-specificity [67]. DICs and

DLICs modulate and regulate the complex activity. More specifically, both mediate cargo binding to dynein, and DICs also regulate the motion by changing motor activity [66, 68].
According to their location, dyneins can be classified as flagellar/axonemal or cytoplasmic (Figure 5) [65, 69]. Flagellar/axonemal dyneins empower the movement of eukaryotic cilia and flagella [68]. Cytoplasmic dyneins associate to diverse protein, RNA-containing complexes, and cellular organelles, such as lysosomes, endosomes and components of the Golgi apparatus, and are responsible for their retrograde transport [68].

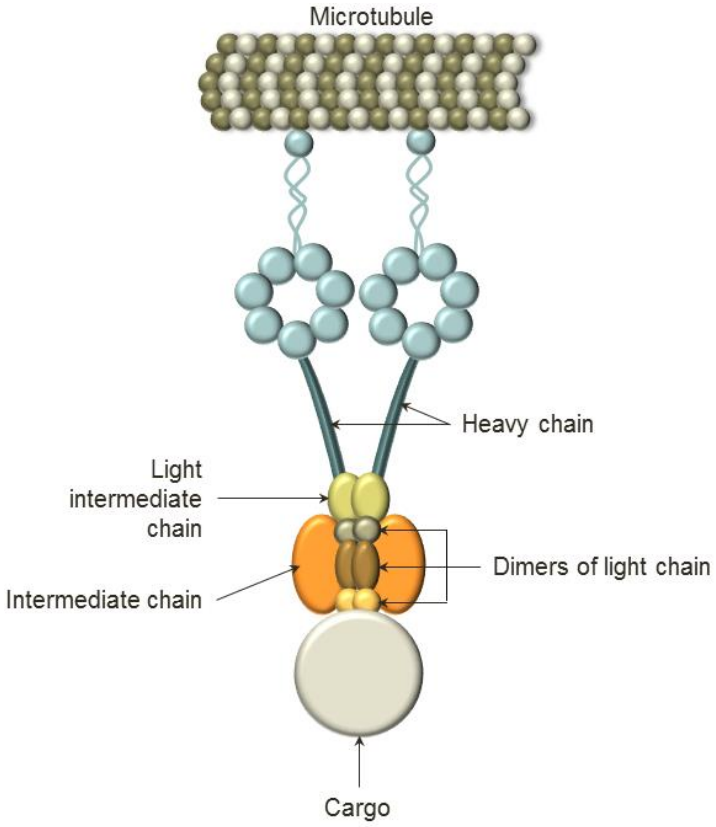


Figure 5 | Organization of cytoplasmic dynein complex.
Dyneins are composed by a homodimer of heavy chains, which forms the motor domain, and smaller accessory subunits (light chain, light intermediate chain, and intermediate chain) with two copies of each.

1.4.2 TCTEX1D4, AN INTERACTOR OF PPP1 AND TGFβ RECEPTORS

DLCs are divided into three families: LC8, LC7/roadblock and TCTEX1/TCTEX2 [69]. TCTEX1D4 is a novel cytoplasmic DLC that belongs to the TCTEX1 protein family. It is expressed in a wide variety of cells and tissues, such as vascular endothelial and smooth muscles cells, placenta, testis, and several tumor cell lines [67].

TCTEX1D4 gene locates in the chromosome 1p34.1 and the gene product has 221 amino acids, being composed by two domains: the disordered domain (residues 1–120) and the globular (residues 121–221) domain, which is common to all family members. The disordered domain presents putative serine phosphorylation sites for several kinases, including for protein kinase A, B and C, cyclin-dependent kinase 1 and glycogen synthase kinase 3 [67, 70].

As observed in the majority of the PIPs, the RVxF motif (RVSF) was found in the residues 90-93 of TCTEX1D4, and evidences suggest that its dephosphorylation is a crucial post-translational modification for TCTEX1D4 regulation [70].

In what concerns TCTEX1D4 functions, the current knowledge is still limited. Besides TCTEX1D4 interaction with PPP1, it was also identified as a partner for receptors of the TGFβ signaling pathway [67]. Actually, TCTEX1D4 can bind to the cytoplasmic domain of endoglin and to TβRII, forming a multimeric complex mainly located at the cell membrane. Interestingly, TCTEX1D4 showed an inhibitory activity on the TGFβ1-induced signaling in liver and lung cells, perhaps because of the blockage of both receptors internalization and their consequent longer retention time in the membrane [67]. Furthermore, it may have a possible role in cell-to-cell junctions and microtubule dynamics. The presence of binding sites for APC/C, cyclins and MAPK also suggests that it possibly play a regulatory role in proliferation, differentiation, and cell cycle [70].

AIMS

The identification of TCTEX1D4 as a PIP and as a TGFβ1-induced signaling inhibitor gave rise to the hypothesis that TCTEX1D4 dephosphorylation by PPP1 may play a critical role in the TGF-β signaling regulation.

Taking into account the role of TGFβ signaling in PCa progression, this study is specifically concerned with the effect of TCTEX1D4 dephosphorylation in such signaling and, consequently, on the cancer cells proliferation and metastasis formation.

Preliminary results from our laboratory confirmed the presence of both TCTEX1D4 and PPP1 in normal and PCa cells. Hence, four human prostate epithelial cell lines will be used: RWPE-1, PNT-2, PC-3, and LNCaP.

To achieve the main goal, the specific aims of this thesis include to:

1. Optimize the growth conditions of human prostate cell lines: RWPE-1, PNT-2, PC-3, and LNCaP;
2. Optimize transfection conditions of RWPE-1, PNT-2, PC-3, and LNCaP cell lines with Myc-TCTEX1D4 and Myc-TCTEX1D4- RVSA;
3. Optimize TGFβ treatment for PNT-2, PC-3, and LNCaP cell lines;
4. Determine the effect of TCTEX1D4/PPP1 in prostate cell proliferation;
5. Determine the role of TCTEX1D4/PPP1 in prostate cell migration.

MATERIALS AND METHODS

All the experiments required to accomplish the aims of this thesis were carried out in the Signal Transduction Laboratory, Center for Cell Biology, University of Aveiro (Aveiro, Portugal).
Additional information about the composition of the solutions and materials used in the experiments is provided in the appendix.

3.1 HUMAN PROSTATE CELL LINES

RWPE-1 (ATCC, Barcelona, Spain) are normal adult human prostate epithelial cells derived from the peripheral zone. PNT-2 cell line (kindly provided by Dr. Ricardo Pérez-Tomás, University of Barcelona, Spain) was established by immortalization of normal adult prostatic epithelial cells, through transfection with a plasmid containing the genome of Simian vacuolating virus 40 (SV40) with a defective replication origin. PNT-2 may be considered a preneoplastic model as they retain many of the features of well-differentiated prostate epithelial cells despite not being completely normal. LNCaP and PC-3 (kindly provided by Dr. Rui Medeiros, Portuguese Institute of Oncology of Porto, Portugal) are human PCa cell lines. LNCaP is a human PCa androgen-sensitive cell line established from

a metastatic lesion in the left supraclavicular lymph node. PC-3 is a highly metastatic and androgen-independent human PCa cell line established from bone metastasis of grade IV. All the cell lines were tested for Mycoplasma and the results were negative.

3.2 WILD TYPE AND MUTANT TCTEX1D4 CDNAS

Myc-TCTEX1D4 (wild type) and Myc-TCTEX1D4-RVSA (mutant) plasmids were already available at the laboratory [64]. Briefly, in Myc-TCTEX1D4, the cDNA of TCTEX1D4 was PCR-amplified and inserted into *EcoRI/XhoI* sites of pCMV-Myc vector (Clontech, Saint Germain-en-Laye, France). Myc-TCTEX1D4-RVSA was created by mutating the last amino acid of the PPP1BM RVSF in the TCTEX1D4 cDNA to an alanine. This resulted in the disruption of the PPP1BM. Empty pCMV-Myc vector (Figure 18 in Appendix) was used as control.

Plasmids were purified according to the "Wizard Plus Maxipreps DNA Purification System" (Promega, Southampton, UK) manufacturer's instruction. Plasmids obtained were stored at -20°C (Table 1).

Table 1 | Plasmids obtained after purification by maxiprep.

For each cDNA, two sets of plasmids were obtained with different concentrations and purities.

Myc-TCTEX1D4		Myc-TCTEX1D4-RVSA	
Concentration	***Purity***	***Concentration***	***Purity***
903.0 ng/µl	1.93	1143.3 ng/µl	1.91
919.3 ng/µl	1.92	1109.5 ng/µl	1.93

3.3 TGFβ1

TGFβ1 (Sigma-Aldrich, Sintra, Portugal) was reconstituted using 0.2 µm filtered 4 mM HCl (Sigma-Aldrich, Sintra, Portugal) containing 1 mg/ml of BSA (Nzytech, Lisboa, Portugal). Stock solution (1 µg/ml) was stored at -20°C.

A pilot experiment was carried out in both serum and serum-free conditions to test the effect of diverse TGFβ1 concentrations in prostate cells proliferation. Given the results, the subsequent experiments were carried out using 5 ng/ml or 20 ng/ml of TGFβ1.

3.4 ANTIBODIES

Primary antibodies used include the PPP1CC1 rabbit polyclonal antibody (homemade, CBC3C), which recognizes the C-terminal sequence of PPP1CC1 isoform, the c-Myc mouse monoclonal antibody (Invitrogen, Madrid, Spain) that recognizes the Myc-tag, and the primary antibody against the C-terminal of TCTEX1D4 (homemade, CBC8C) (Table 3 in Appendix).

Secondary antibodies used include Texas Red goat anti-mouse (Invitrogen, Madrid, Spain) and Infrared IRDye-labeled anti-rabbit specific to Li-Cor's Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany) (Table 3 in Appendix).

3.5 CELL LINES MAINTENANCE

RWPE-1 were cultured in Keratinocyte Serum Free Medium (K-SFM; Gibco, Madrid, Spain) supplemented with 5 ng/ml of epidermal growth factor (EGF; Gibco, Madrid, Spain), 0.05 mg/ml of bovine pituitary extract (BPE; Gibco, Madrid, Spain) and 1% (v/v) penicillin/streptomycin/amphotericin solution (Gibco, Madrid, Spain). PNT-2, LNCaP, and PC-3 were cultured in RPMI-1640 Medium (Gibco, Madrid, Spain) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Madrid, Spain) and 1% (v/v) penicillin/streptomycin amphotericin solution. The cell lines were maintained in a 5% CO_2 humidified incubator at 37°C. Media renewal occurred every 2–3 days.

3.6 THAWING CELLS AND CRYOPRESERVATION

Complete growth media were previously placed in the incubator for 15 minutes. A vial of frozen cells was retrieved from liquid nitrogen freezer. The vial was rapidly thawed by gently agitation in a water bath set at 37°C. Cells were immediately centrifuged at 1000 rpm, 22°C, for 3 minutes (LNCaP and PC-3) or for 7 minutes (RWPE-1). Pellet was then resuspended in 1 ml of complete growth medium, transferred to a 60 mm culture dish (VWR International, Carnaxide, Portugal), and incubated in a 5% CO_2 humidified incubator at 37°C.

For cryopreservation, cells were resuspended in cryopreservation medium (complete growth medium supplemented with 5% (v/v) dimethyl sulfoxide (DMSO)).

3.7 TRYPAN BLUE ASSAY FOR CELL PLATING

In order to plate the same amount of cells per well, cells were initially counted using the dye exclusion assay Trypan Blue (Sigma-Aldrich, Sintra, Portugal). Briefly, the principle of this assay lies on the fact that living cells with an intact cytoplasmic membrane do not incorporate the reagent, while dead cells retain the dye and stain blue.

Briefly, 10 μl of 0.4% Trypan Blue were added to 90 μl of cell suspension. The unstained (viable) cells were counted in a Neubauer chamber and cell concentration was calculated for cell plating.

3.8 COATING OF PLATES AND COVERSLIPS FOR CELL ATTACHMENT

Before seeding cells, an 18 mm coverslip was introduced in each well of a 6-well plate. Poly-L-ornithine (Sigma-Aldrich, Sintra, Portugal), 0.1 mg/ml, was added to each well for 5 minutes. At that point, poly-L-ornithine was removed and the plate was allowed to dry. The plates were then washed with autoclaved water and completely dry before use.

3.9 TRANSIENT TRANSFECTION WITH LIPOFECTAMINE 2000

To assess the effect of TCTEX1D4/PPP1 complex in cell proliferation and migration, human prostate cell lines were transiently transfected with the cDNAs mentioned above using Lipofectamine 2000 (Invitrogen, Madrid, Spain) transfection reagent.

Lipofectamine 2000 is a cationic-lipid transfection reagent that complexes with negatively charged nucleic acids. The positive charge of liposomes masks the negative charge of nucleic acids, enabling their interaction with the cell membrane and resultant endocytosis of the complexes into the cytoplasm.

A pilot experiment was performed to select the most accurate conditions for human prostate cells transient transfection. Different amounts of DNA and transfection times were tested following the manufacturer's instructions. Transfection efficiency (TE) was assessed by Western blot and immunofluorescence analysis.

In order to have 70-90% confluence at the time of transfection, cells ($5x10^5$/well) were seeded in 6-well plates with complete growth medium (2 ml/well), 24 hours prior to transfection. At the time of transfection, cell medium was changed to 2 ml/well of Opti-

MEM Reduced Serum Medium (Invitrogen, Madrid, Spain). To proceed with transfection, 3 µg of DNA were diluted in 250 µl of Opti-MEM Reduced Serum Medium. After being gently mixed, 6 µl of Lipofectamine 2000 were diluted in 250 µl of Opti-MEM Reduced Medium and incubated for 5 minutes at room temperature (RT). Next, the diluted DNA was added to the diluted Lipofectamine 2000. The mixture was incubated for 25 minutes at RT to allow complexes formation. The 500 µl of transfection mix was added dropwise to each well and the plates were gently agitated. Then, the 6-well plates were incubated at 37ºC in a humidified incubator in an atmosphere of 5% CO_2. After 4 hours of incubation, 2 ml of growth medium without antibiotics were added to each well and the plates returned to the incubator to complete 24 hours of transfection.

3.10 CELL COLLECTION AND QUANTIFICATION OF PROTEIN CONTENT

Cell medium was removed and cells were collected with 150 µl of 1% boiling sodium dodecyl sulfate (SDS; Sigma-Aldrich, Sintra, Portugal). Cell lysates were boiled for 10 minutes and sonicated for 15 seconds. The total protein content of the samples was then determined by the Pierce's bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Bremen, Germany), following the manufacturer's instructions.

The principle of this assay relies on two subsequent chemical reactions: (1) the biuret reaction that involves the reduction of Cu^{2+} to Cu^{+}, being the amount of Cu^{2+} reduced proportional to the quantity of protein present in the solution; (2) the chelation of two molecules of BCA with each Cu^{+} ion primarily formed, resulting in the formation of a purple-colored water-soluble complex that absorbs light at a wavelength of 562 nm. The absorbance at 562 nm is linear with increasing protein concentration over a working range of 20-2000 µg/ml. Total protein concentration of each sample was determined through a standard curve prepared by plotting bovine serum albumin (BSA) absorbance vs. BSA standard concentration. The standards protein concentrations were prepared in a 96-well plate as described in Table 4 (Appendix).

Each sample was prepared, in a well of the 96-well plate, by adding 5 µl of the sample to 20 µl of 1% SDS. The working reagent (WR) was prepared by mixing the BCA reagent A with BCA reagent B in a 50:1 proportion, and 200 µl of this mixture were added to both standards and samples wells. The 96-well plate was incubated at 37ºC for 30 minutes.

After briefly cooled at RT, the absorbance at 562 nm was immediately measured using the microplate reader Infinite M200 (Tecan, Barcelona, Spain).

3.11 WESTERN BLOTTING

After mass normalization, samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), which enables the migration of proteins according to their molecular weight, and were subsequently transferred into nitrocellulose membranes.
Samples were prepared by adding 1% SDS and 4X loading buffer to the protein content of each sample, and run in a 15% gel at 200 V (composition of the gels are stated in appendix). Afterward, proteins were electrotransferred onto nitrocellulose membranes at 200 mA. The resultant membranes were hydrated in 1X tris buffered saline (TBS) for 5 minutes and blocked with a solution of 5% non-fat milk in 1X TBS with tween (TBST) for 1 hour. Membranes were then incubated with the primary antibody (diluted in 3% non-fat milk/1X TBST) for 2 hours, washed three times in 1X TBST, and incubated with the appropriate secondary antibody (also diluted in 3% non-fat milk/1X TBST) for 1:30 hours. After two washing steps with 1X TBST and one with 1X TBS, 10 minutes each, membranes were scanned in Li-Cor's Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

3.12 IMMUNOFLUORESCENCE

Coverslips were transferred to a new 6-well plate with 1 ml/well of 1X phosphate buffered saline (PBS; Thermo Scientific, Bremen, Germany). PBS was removed and 1 ml of 4% paraformaldehyde was added for 30 minutes. After two washing steps with 1X PBS, coverslips were permeabilized in 500 µl of 0.2 TRITON X-100 (diluted in 1X PBS) for 10 minutes, and blocked in 50 µl of 1X PBS–3% BSA for 30 minutes. The blocking solution was removed and coverslips were incubated with 50 µl of the primary antibody, previously diluted in 1X PBS–3% BSA, for 2 hours, and then with the respective secondary antibody, previously diluted in 1X PBS–3% BSA, for 1 hour. After addition of the secondary antibody, procedures were carried out in a dark environment. Coverslips were mounted

onto microscope slides using Vectashield Mounting Medium with DAPI (Vector Laboratories, Lisboa, Portugal) and sealed for microscope visualization.
Images were acquired by epifluorescence microscopy using an Olympus IX-81 motorized inverted microscope equipped with LCPlanFl 20x/0.40 objective lens.

3.13 PROLIFERATION ASSAY

Cell proliferation was measured with the cell viability indicator alamarBlue (AB; Invitrogen, Madrid, Spain), used for quantitative measurement of cell viability and cytotoxicity. The active component of AB is resazurin, a nontoxic, cell permeable, and almost non-fluorescent compound. Viable cells uptake resazurin and reduce it, continuously, to resorufin, a very bright red fluorescence compound. Henceforth, the fluorescence and color of the cell medium is proportional to the extent of the reaction and consequently to the number of living cells.
Cells were trypsinized and centrifuged at 1000 rpm for 3 minutes at 22°C. After cell resuspension in serum-free medium, cells were counted as s
ated in 3.4., and $2x10^3$ cells/well were plated in a 96-well plate containing 100 µl/well of the same growth medium. Cells were incubated in a 5% CO_2 humidified incubator at 37°C for 4 hours to let cells adhere. Afterward, TGFβ1 (5 or 20 ng/ml) were added to each well or, as controls, cells were left untreated or were treated with the TGFβ1 vehicle solution (0.2 µm filtered 4 mM HCl with 1 mg/ml of BSA). The plate returned to the incubator for an additional period of 24 hours.
AB was then aseptically and directly added into the culture medium at a final concentration of 10%. Blanks consisted of only medium with 10% AB. Absorbance at both 570 nm and 600 nm was read 4, 12, 24 and 36 hours microplate reader Infinite M200 (Tecan, Barcelona, Spain). Percentage of AB reduction was calculated as depicted on Figure 6.

$$\text{Percentage of reduction of AB} = \frac{(O2 \text{ x } A1) - (O1 \text{ x } A2)}{(R1 \text{ x } N2) - (R2 \text{ x } N1)} \text{ x } 100$$

Figure 6 | Equation applied to calculate the percentage of reduction of AB.
O1: Molar extinction coefficient (E) of oxidized AB (Blue) at 570nm; O2: E of oxidized AB at 600nm; R1: E of reduced AB (Red) at 570nm; R2: E of reduced AB at 600nm; A1: Absorbance of test wells at 570nm; A2: Absorbance of test wells at 600nm; N1: Absorbance of negative control well at 570nm; N2: Absorbance of negative control well at 600nm.

3.14 MIGRATION ASSAY

Cell migration was assessed using HTS Transwell-96 Permeable Support Plates (VWR International, Carnaxide, Portugal) with a polyester membrane with 8.0 μm pore size (Figure 19 in Appendix).

Transfected cells were trypsinized and centrifuged at 1000 rpm for 3 minutes at 22°C. After cell resuspension in serum-free medium, cells were counted as stated in 3.4., and $2.5x10^3$ cells/well of cell suspension were seeded on the top of the polyester membranes in the seeder plate. The seeder plate was immersed in a reservoir plate fulfilled with 150 μl of serum-free medium and plates were incubated in a 5% CO_2 humidified incubator at 37°C. After 4 hours of initial cell attachment, 5 or 20 ng/ml of TGFβ1 were added to the reservoir plate, or cells were left untreated. The plate returned to the incubator for an incubation period of 24 hours to let cells migrate through the polyester membrane.

At this time, cells that did not migrate were removed from the seeder plate with a cotton swab. AB was then aseptically added into the culture medium in the reservoir plate at a final concentration of 10%. Blanks consisted of only medium with 10% AB Absorbance at both 570 nm and 600 nm was read 24 hours microplate reader Infinite M200 (Tecan, Barcelona, Spain).

3.15 DATA ANALYSIS

Absorbance measurements were used to calculate the percentage of reduction of AB. Data were presented as mean ± standard deviation (SD) or mean ± 95% confidence interval (the chosen error bar was stated in the legend for each case).

Significant differences between median proliferation and migration rates of the different groups (non-transfected cells, TCTEX1D4 transfected cells, and TCTEX1D4-RVSA transfected cells) were evaluated with the Kruskal–Wallis one-way analysis of variance test (α=0.05).The same test was used to assess significant differences between the groups in response to TGFβ treatment.

This first approach was followed by multiple comparisons using the Mann-Whitney U test with Bonferroni correction (α=0.017) to compare TCTEX1D4 and TCTEX1D4-RVSA

groups versus non-transfected cells and also TCTEX1D4 versus TCTEX1D4-RVSA transfected cells. IBM SPSS Statistics 21 software was used to compute all tests.

RESULTS

The initial experiments of optimization were performed using RWPE-1, LNCaP, and PC-3 cells. After a kind donation from Dr. Ricardo Pérez-Tomás, we started to use PNT-2 cells as a benign control in the experiments.

4.1 TCTEX1D4 AND PPP1 ARE PRESENT IN HUMAN PROSTATE CANCER CELLS

TCTEX1D4 expression was already described in several tissues, including ovary, spleen, lung, placenta, and kidney [70]. Its presence was also observed in a number of cell lines, as human umbilical vein endothelial cells (HUVECs), human dermal microvascular endothelial cell line (HMEC-1), hepatocellular carcinoma cells (HepG2), breast cancer cells (T47D), and large cell lung cancer (LCLC) [67].

To confirm the presence of TCTEX1D4 in human prostate cells, a preliminary experiment was performed using a polyclonal antibody, raised in rabbit, against the C-terminal of TCTEX1D4 (CBC8C, Appendix). In LNCaP and PC-3 cells we observed a band at around 28 kDa, corresponding to the full-length TCTEX1D4 (Figure 7). The protein predicted molecular weight, nonetheless, is of 23.4 kDa. This band shift might be a consequence of

post-translational modifications, such as phosphorylation and/or glycosylation as already mentioned by Korrodi *et. al* [70]. The expression of TCTEX1D4 in normal prostate cells RWPE-1 was only detectable after protein concentration by immunoprecipitation (data not shown).

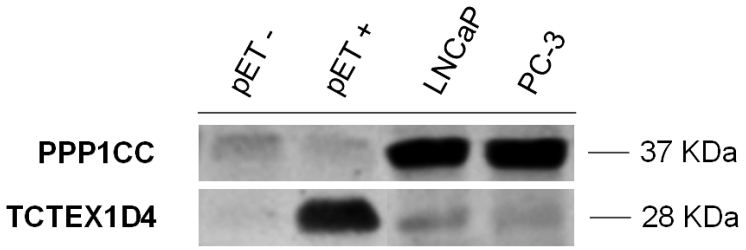


Figure 7 | TCTEX1D4 and PPP1CC are present in human PCa cell lines LNCaP and PC-3.
The screening (100 µg) of TCTEX1D4 and PPP1CC isoform was performed using CBC8C and CBC3C antibodies, respectively. Extracts were prepared in 1% SDS. pET vector alone (pET-) was used as negative control and pET-TCTEX1D4 (pET+) was used as positive control.

Despite the fact that PPP1 is a ubiquitously expressed protein, we also assessed the expression of PPP1CC in the same immunoblot. The blot was incubated with a polyclonal antibody, raised in rabbit, against the C-terminal of PPP1CC isoform (CBC3C). A band was detected at 37 kDa, in both cell lines, according to the PPP1 predicted molecular weight (Figure 7). Additionally, PPP1CA expression in these cell lines was also confirmed by Western blot and both PPP1 isoforms were also observed in RWPE-1 and PNT-2 (data not shown).

4.2 OPTIMIZATION OF THE TRANSFECTION METHOD

The interaction between TCTEX1D4 and PPP1 was previously established in a testis yeast-two hybrid and was further confirmed by yeast co-transformation and co-immunoprecipitation [70]. Once we have confirmed the presence of both TCTEX1D4 and PPP1 in human prostate cells, we focused our attention in the possible influence of this complex in cell proliferation and migration. To achieve the main objectives, we transfected human prostate cells with Myc-TCTEX1D4 or Myc-TCTEX1D4-RVSA plasmids. Hence, a first approach aimed to optimize cell transfection conditions.

A pilot experiment was performed using different amounts of DNA (1µg, 2µg, and 3µg) and Lipofectamine 2000 (2µl, 4µl, and 6µl) and also different times of transfection (8 and 24 hours) in low-passage RWPE-1, LNCaP and PC-3 cells. The TE obtained was assessed both by Western blot and immunofluorescence.

Results obtained when cells were transfected with 1 µg of Myc-TCTEX1D4 or Myc-TCTEX1D4-RVSA to 2 µl of Lipofectamine 2000, as well as when transfection occurred for 8 hours, are not shown due to the few transfected cells observed. For the other cases, blots showed a band at around 28 kDa that corresponds to the TCTEX1D4 expressed by transfected cells 24 hours after transfection (Figure 8).

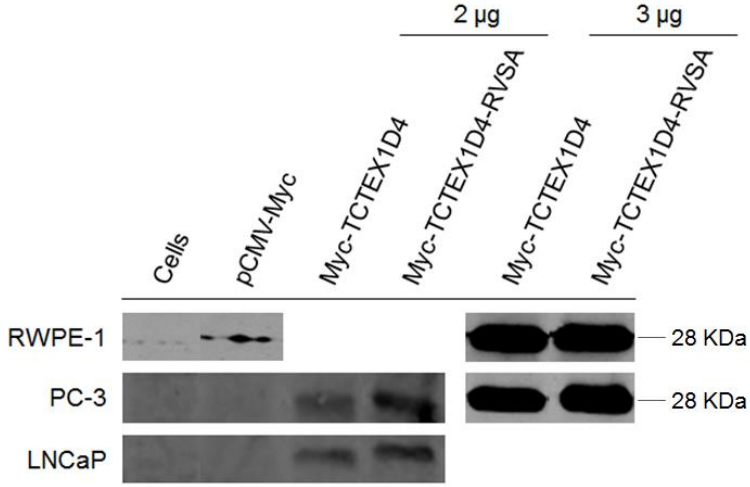


Figure 8 | Immunoblot analysis of Myc-TCTEX1D4 and Myc-TCTEX1D4-RVSA transfection in RWPE-1, PC-3, and LNCaP cells using different amounts of DNA and Lipofectamine 2000 (ratio 1:2).

Cells were transfected for 24 hours using Lipofectamine 2000. Extracts were prepared in 1% SDS and 50 µg of protein were run in a 15% polyacrylamide gel. Blots were incubated with mouse anti-Myc-tag antibody. Cells alone and cells transfected with the empty vector pCMV-Myc were used as negative controls.

This analysis was complemented with a quantitative study by immunofluorescence. In this case, PNT-2 cell line was already available to be included in the experiment. Cells were stained with the c-Myc mouse monoclonal primary antibody and the Texas Red goat anti-mouse secondary antibody; nuclei were stained with DAPI. The number of transfected cells was estimated using an epifluorescence microscope, under a magnification of 20x.

The best TE was achieved when cells were transfected for 24 hours with 3 µg of DNA to 6µl of Lipofectamine 2000, for both Myc-TCTEX1D4 and Myc-TCTEX1D4-RVSA plasmids (Figure 9). For this reason, all the subsequent experiments were carried out under these conditions.

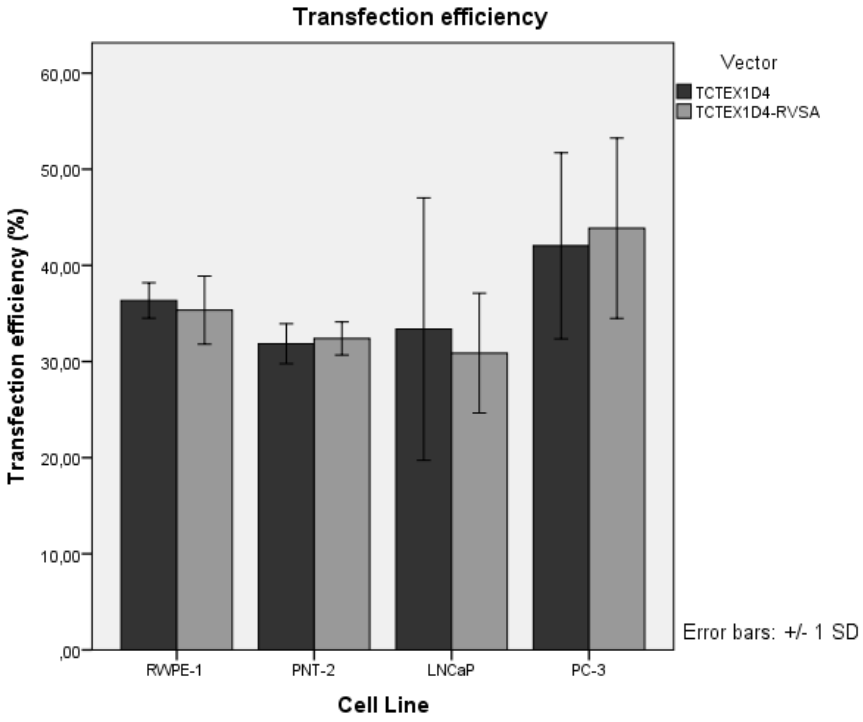
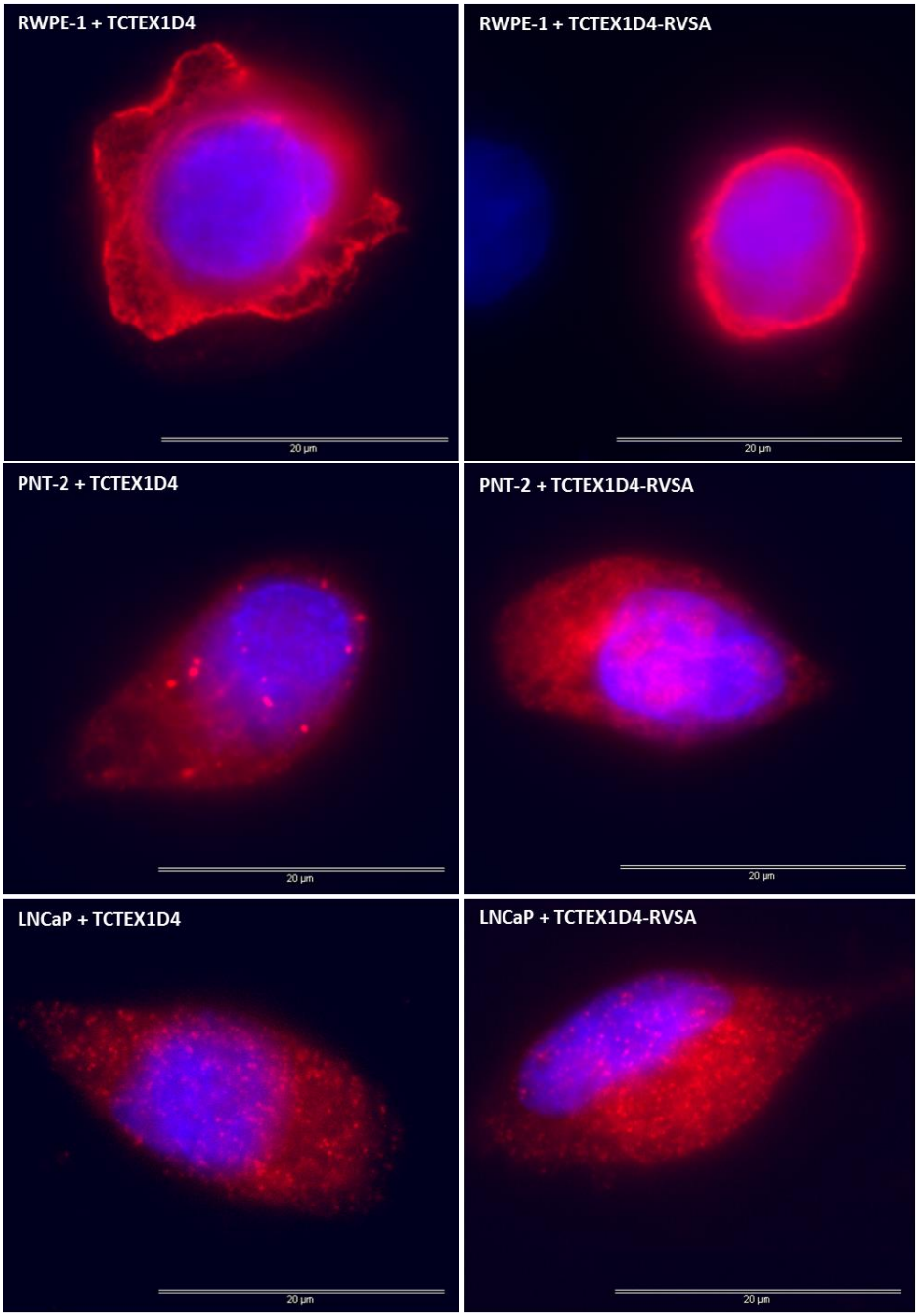


Figure 9 | Transfection efficiency for each vector (TCTEX1D4/TCTEX1D4-RVSA) in each cell line.

Cells were transfected with 3 µg of DNA and 6 µl of Lipofectamine 2000 for 24h. The graph shows the average number of transfected cells determined by fluorescence microscopic visualization. Data represent the mean ± standard deviation (SD) of three independent experiments. RWPE-1 TCTEX1D4: 36.35±1.85%; RWPE-1 TCTEX1D4-RVSA: 35.34±3.54%; PNT-2 TCTEX1D4: 31.85±2.07%; PNT-2 TCTEX1D4-RVSA: 32.40±1.72%; LNCaP TCTEX1D4: 33.37±13.65%; LNCaP TCTEX1D4-RVSA: 30.87±6.23%; PC-3 TCTEX1D4: 42.04±9.68%; PC-3 TCTEX1D4-RVSA: 43.87±9.38%.

4.3 IMMUNOFLUORESCENCE ANALYSIS OF TRANSFECTED CELLS

The immunofluorescence analysis also enabled the comparison of transfected TCTEX1D4 and mutant TCTEX1D4 localization and distribution (Figure 10).



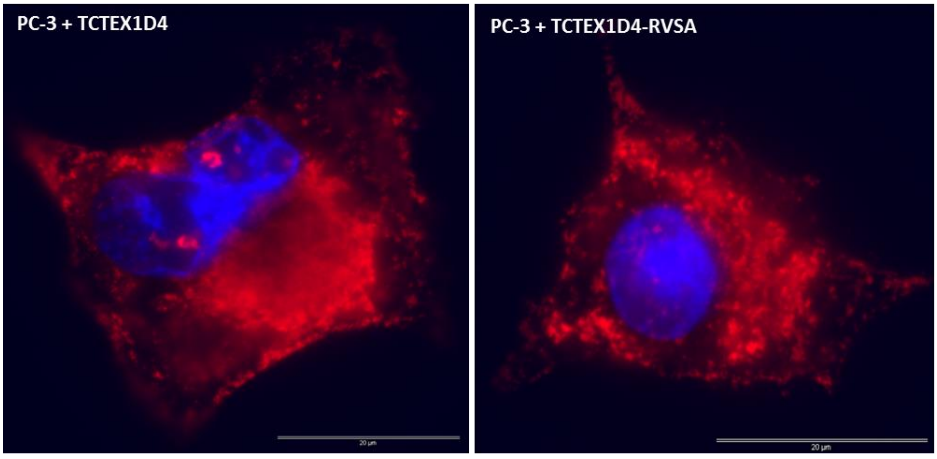


Figure 10 | Fluorescence microscopy visualization and intracellular distribution of Myc-TCTEX1D4 and Myc-TCTEX1D4-RVSA in transfected cells.

RWPE-1, PNT-2, PC-3, and LNCaP cells were transfected with Myc-TCTEX1D4 or Myc-TCTEX1D4-RVSA (red), labeled with c-Myc mouse monoclonal antibody and Texas Red anti-mouse secondary antibody. Nuclei (blue) were stained with DAPI. Images were analyzed by an epifluorescence microscope. Scale bars:20 µm.

Outwardly, there is no significant difference between the distribution of wild type and mutant TCTEX1D4. But interestingly, the stained pattern of RWPE-1 cells is distinct when compared to the other cell lines. The subcellular localization of transfected TCTEX1D4/TCTEX1D4-RVSA in PNT-2, PC-3, and LNCaP was dispersed in the cell nucleus and cytoplasm. Transfected TCTEX1D4/TCTEX1D4-RVSA in RWPE-1 appears to be mainly confined to the perinuclear space and cytoplasmic membrane.

4.4 ESTABLISHMENT OF TGFβ1 CONCENTRATIONS FOR CELL TREATMENT

The next step in our workflow aimed to establish the ideal concentrations of TGFβ1 to treat human prostate cells. To start with, we revised the literature available in PubMed, using the terms "transforming-growth factor beta 1", "prostate", and "cell proliferation" as keywords (Table 2).

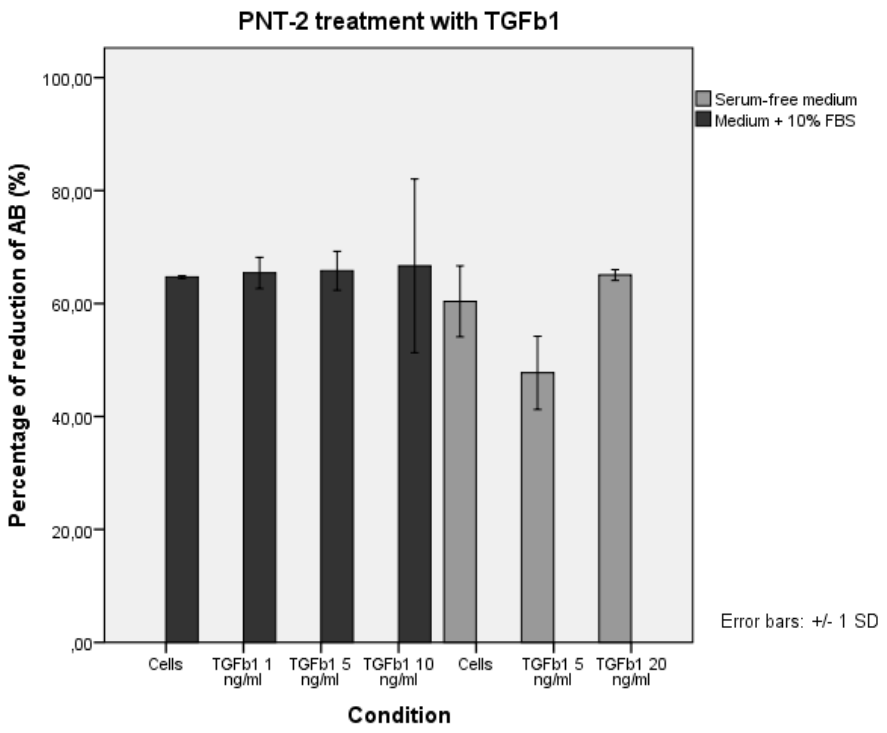
Table 2 | Summary of the information found during the PubMed search.

Cell line	TGFβ1 concentration	Cellular effect
RWPE-1	10 ng/ml (serum-free, 8h)	Proliferation: decreased proliferation [71].
PNT-2	1, 2, or 5 ng/ml (serum-free, 48h)	Proliferation: dose-dependent growth inhibition [72].
LNCaP	1 ng/ml (48h)	Migration: no effect [73].
	1 ng/ml (5% FBS, 18h)	Proliferation: no inhibitory effect. Instead, there was a slight increase comparing to the controls [74].
	10 ng/ml (5% FBS, 18h)	Proliferation: no effect [74].
PC-3	1 ng/ml (48h)	Migration: increased migration [73].
	1 ng/ml (48h)	Proliferation: no inhibitory effect. Instead, there was a slight increase comparing to the controls [73].
		Migration: Increased migration [74].
	5 ng/ml (48h)	Migration: increased migration [74].

Taking into account this information, we performed a pilot experiment to test different conditions. The first attempt was carried out in medium supplemented with 10% FBS. Cells were treated with different concentrations of TGFβ1 (1 ng/ml, 5 ng/ml, and 10 ng/ml) for 48 hours and, afterward, cell proliferation was measured using AB. The percentage of reduction of AB, and consequently the cell proliferation, was calculated by applying the equation on Figure 6.

Due to the virtually no effect of TGFβ1 in cell proliferation, a next try was accomplished in serum-free medium with a lower cell plating density using a low (5 ng/ml) and a high (20 ng/ml) concentration of TGFβ1. Cell proliferative behavior in each condition is depicted in Figure 11.

A



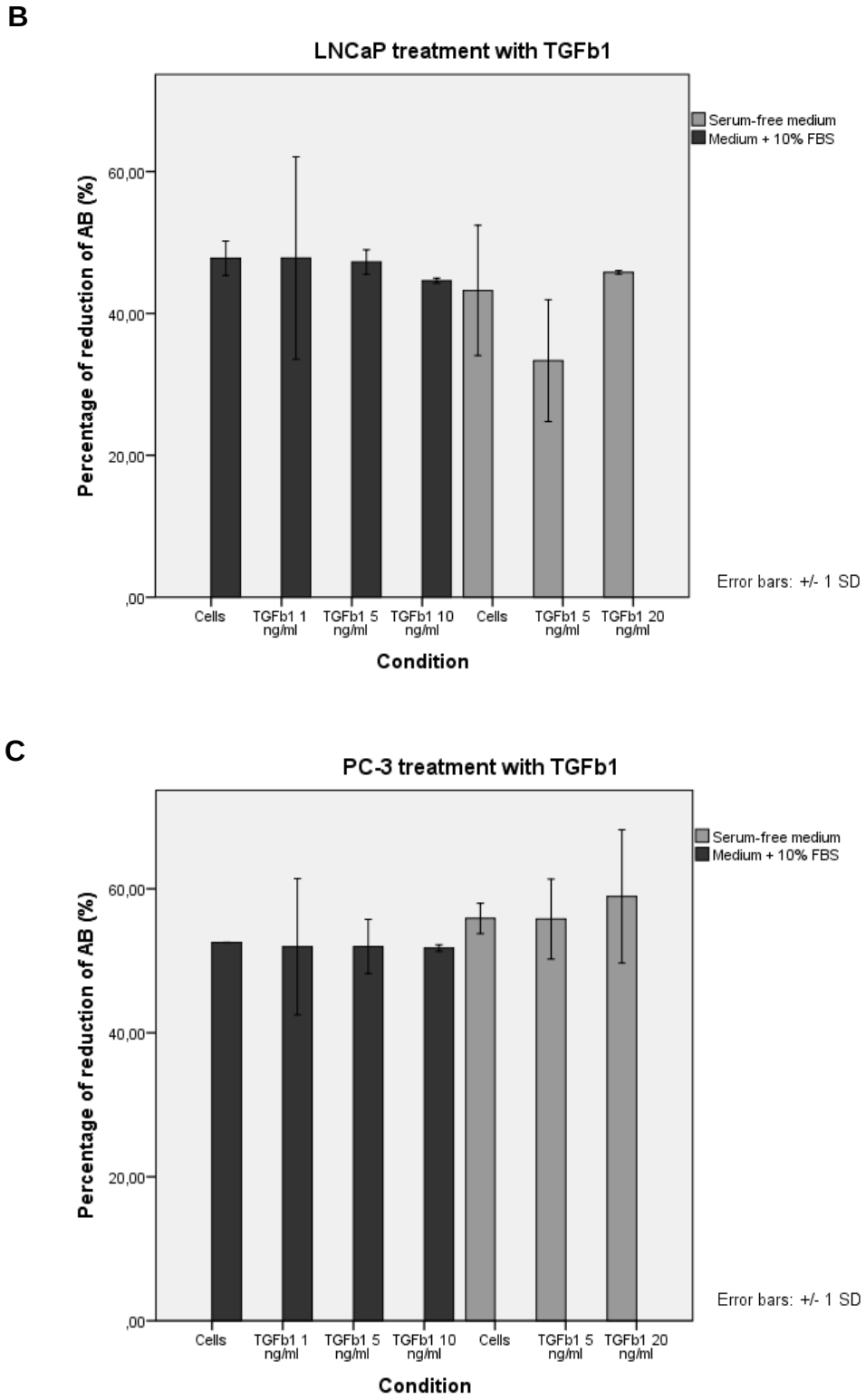


Figure 11 | Proliferation of PNT-2, LNCaP, and PC-3 cells in response to TGFβ1.

Cells were seeded in medium supplemented with 10% FBS or in serum-free medium. Each cell line was exposed to a 24 hours treatments with different concentrations of TGFβ1. Cells with no treatment were used as negative control. The graph shows the average percentage of reduction of AB for PNT-2 (A), LNCaP (B), and PC-3 (C). Data represent the mean ± SD for two replicates of each condition. Mean values ± Standard Deviation were as follows: A) Medium + 10%

FBS – Cells: 64.69±0.25%; TGFβ1 1 ng/ml: 65.42±2.76%; TGFβ1 5 ng/ml: 65.80±3.46%; TGFβ1 10 ng/ml: 66.68±15.37%. Serum-free medium – Cells: 60.39±6.29%; TGFβ1 5 ng/ml: 47.73±6.48%; TGFβ1 20 ng/ml: 65.05±0.95%. B) Medium + 10% FBS – Cells: 47.78±2.43%; TGFβ1 1 ng/ml: 47.82±14.28%; TGFβ1 5 ng/ml: 47.26±1.73%; TGFβ1 10 ng/ml: 44.63±0.36%. Serum-free medium – Cells: 43.24±9.18%; TGFβ1 5 ng/ml: 33.34±8.58%; TGFβ1 20 ng/ml: 45.79±0.24%. C) Medium + 10% FBS – Cells: 52.54±0.02%; TGFβ1 1 ng/ml: 51.96±9.47%; TGFβ1 5 ng/ml: 51.99±3.76%; TGFβ1 10 ng/ml: 51.78±0.45%. Serum-free medium – Cells: 55.89±2.11%; TGFβ1 5 ng/ml: 55.80±5.56%; TGFβ1 20 ng/ml: 58.95±9.26%.

Given these results, the subsequent experiments were performed under a serum-free environment with 5 ng/ml or 20 ng/ml of TGFβ1.

4.5 PROLIFERATION OF TRANSFECTED CELLS IN RESPONSE TO TGFβ1

In furtherance of determine the effect of TCTEX1D4/PPP1 complex in human prostate cells proliferation, cells were transfected with wild type TCTEX1D4 or a mutant form that present an alteration in a residue of the PPP1 binding domain. This alteration (from RVSF to RVSA) allows a 35% diminishment of PPP1 binding to TCTEX1D4.

Transfected cells where subjected to a 48 hours treatment with TGFβ (5 ng/ml or 20 ng/ml). Non-transfected cells either with no treatment or treated with the vehicle (solution where TGFβ were reconstituted) were used as negative controls. Cell proliferation was monitored with the cell dye AB at several times of the experiment upon the addition of TGFβ1. The results showed the mean percentage of reduction of AB for three independent experiments (each of three replicates) at the end-point of the experiment (48 hours).

Inside cells, AB is reduced in by mitochondrial enzymes, as flavin mononucleotide dehydrogenase, flavin adenine dinucleotide dehydrogenase, nicotinamide adenine dehydrogenase, and cytochromes, and also by cytosolic and microsomal enzymes. Resofurin, a red-fluorescent compound, is excreted to the medium, resulting in color change from blue to pink. Thus, the rate of reduction based on color changes reflects the number of viable cells [75].

4.5.1 PNT-2

As depicted in Figure 12, both TCTEX1D4 and TCTEX1D4-RVSA transfected cells presented a lower proliferation rate (55.35% and 40.96%, respectively) when compared with non-transfected cells (71.47%). Kruskal-Wallis test confirmed the existence of statistically significant differences p=0.009.

The addition of TGFβ1 did not produced significant effects in any of the conditions. However, the addition of TGFβ1 to the TCTEX1D4 transfected cells decreased the proliferation rate (to 50.89% and 69.16%, depending on the concentration) when compared to the vehicle control (93.13%). The same occurred in a lesser extent in TCTEX1D4-RVSA transfected cells, where the vehicle control presented a proliferation of 49.72% and cells treated with TGFβ1 decreased the proliferation in a dose-dependent manner to 44.0% and 38.92%.

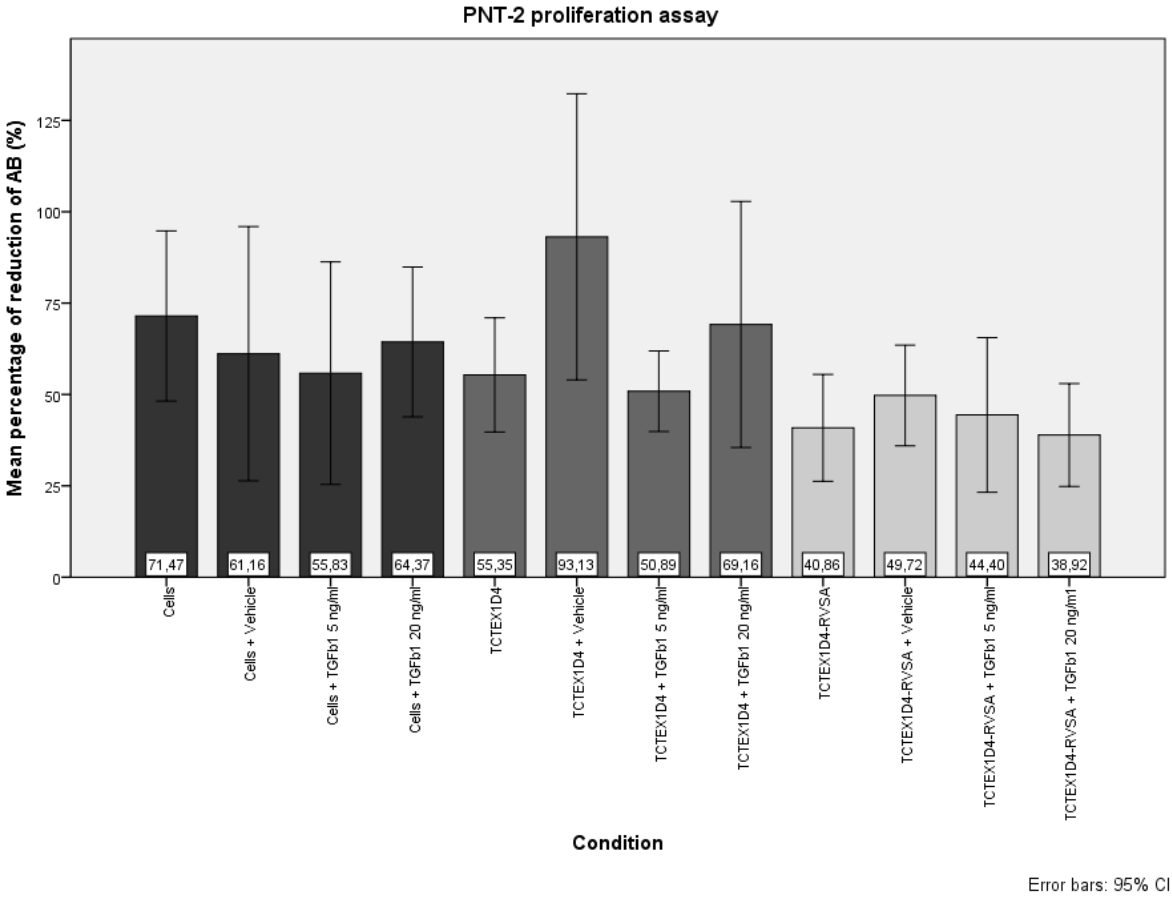


Figure 12 | Proliferation of PNT-2 transfected cells in response to TGFβ1.

Cells were treated with TGFβ1 (5 ng/ml or 20 ng/ml), left untreated or treated with the vehicle for 48 hours. The graph shows the average percentage of reduction of AB. Data represent the mean and 95% confidence intervals (95% CI) for three independent experiments, each of three replicates for each condition. Mean value is displayed for each condition. Mean values ± Standard Deviation were as follows: Cells: 71.47±30.26%; cells + vehicle: 61.16±45.26; cells + TGFβ1 5 ng/ml: 55.83±39.64; cells + TGFβ1 20 ng/ml: 64.37±26.68%; TCTEX1D4: 55.35±20.31; TCTEX1D4 + vehicle: 93.13±37.32; TCTEX1D4 TGFβ1 5 ng/ml: 50.89±14.36; TCTEX1D4 TGFβ1 20 ng/ml: 69.16±43.77; TCTEX1D4-RVSA: 40.86±19.04; TCTEX1D4-RVSA + vehicle: 49.72±13.13 ;TCTEX1D4-RVSA TGFβ1 5 ng/ml: 44.40±27.52; TCTEX1D4-RVSA TGFβ1 20 ng/ml: 38.92±18.31.Differences in the variable "transfected protein" were statistically significant (p = 0.009).

4.5.2 LNCaP

As can be observed in Figure 13, LNCaP cells transfected with TCTEX1D4 or TCTEX1D4-RVSA presented a lower proliferation rate (41,62% and 32,53%, respectively) when compared to non-transfected cells (52,25%). However, differences were not statistically significant.

TGFβ1 treatment narrowed down the proliferation of cells in all conditions when compared with the vehicle control, but not in a significant way.

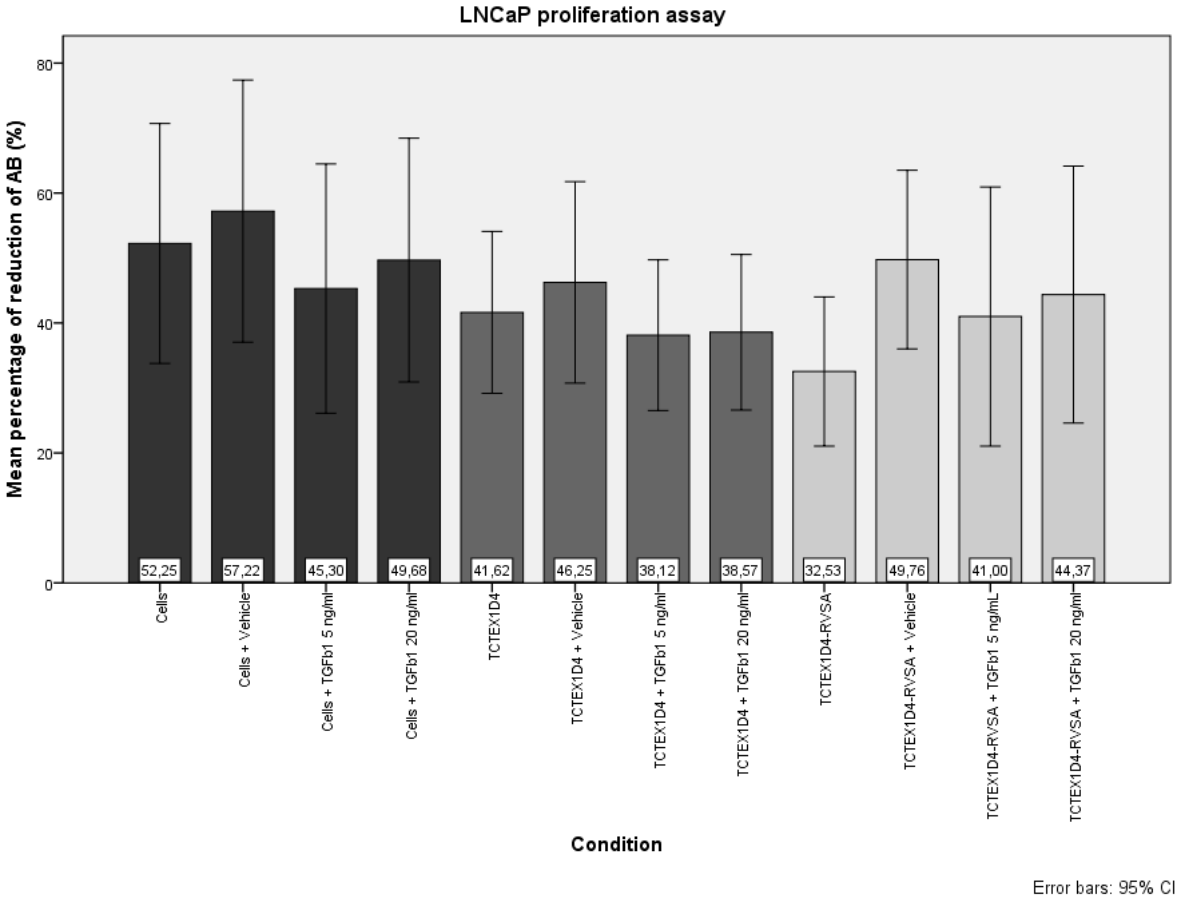


Figure 13 | Proliferation of LNCaP transfected cells in response to TGFβ1.

Cells were treated with TGFβ1 (5 ng/ml or 20 ng/ml), left untreated or treated with the vehicle for 48 hours. The graph shows the average percentage of reduction of AB. Data represent the mean and 95% confidence intervals (95% CI) for three independent experiments, each of three replicates for each condition. Mean value is displayed for each condition. Mean values ± Standard Deviation were as follows: Cells: 52.25±24.026%; cells + vehicle: 57.22±26.25; cells + TGFβ1 5 ng/ml: 45.30±24.95; cells + TGFβ1 20 ng/ml: 49.68±24.41%; TCTEX1D4: 41.62±16.21; TCTEX1D4 + vehicle: 46.25±14.78; TCTEX1D4 TGFβ1 5 ng/ml: 38.12±15.08; TCTEX1D4 TGFβ1 20 ng/ml: 38.57±15.58; TCTEX1D4-RVSA: 32.53±14.94; TCTEX1D4-RVSA + vehicle: 49.76±13.09 ;TCTEX1D4-RVSA TGFβ1 5 ng/ml: 41.00±25.94; TCTEX1D4-RVSA TGFβ1 20 ng/ml: 44.37±25.73.

4.5.3 PC-3

As depicted in Figure 14, PC-3 cells transfected with TCTEX1D4 or TCTEX1D4-RVSA presented a lower proliferation rate (38.64% and 34.34%, respectively) when compared to non-transfected cells (57.38%). Kruskal-Wallis test confirmed the existence of statistically significant differences $p<0.001$.

The addition of TGFβ1 produced almost no effect in non-transfected cells. In TCTEX1D4 transfected cells, nevertheless, it decreased cell proliferation capability (38.15% and

43.11%, depending on concentration) when compared to the vehicle control (52.59%). In TCTEX1D4-RVSA transfected cells, high concentrations of TGFβ1 appeared to induce cell proliferation. However, any of the differences observed were statistically significant.

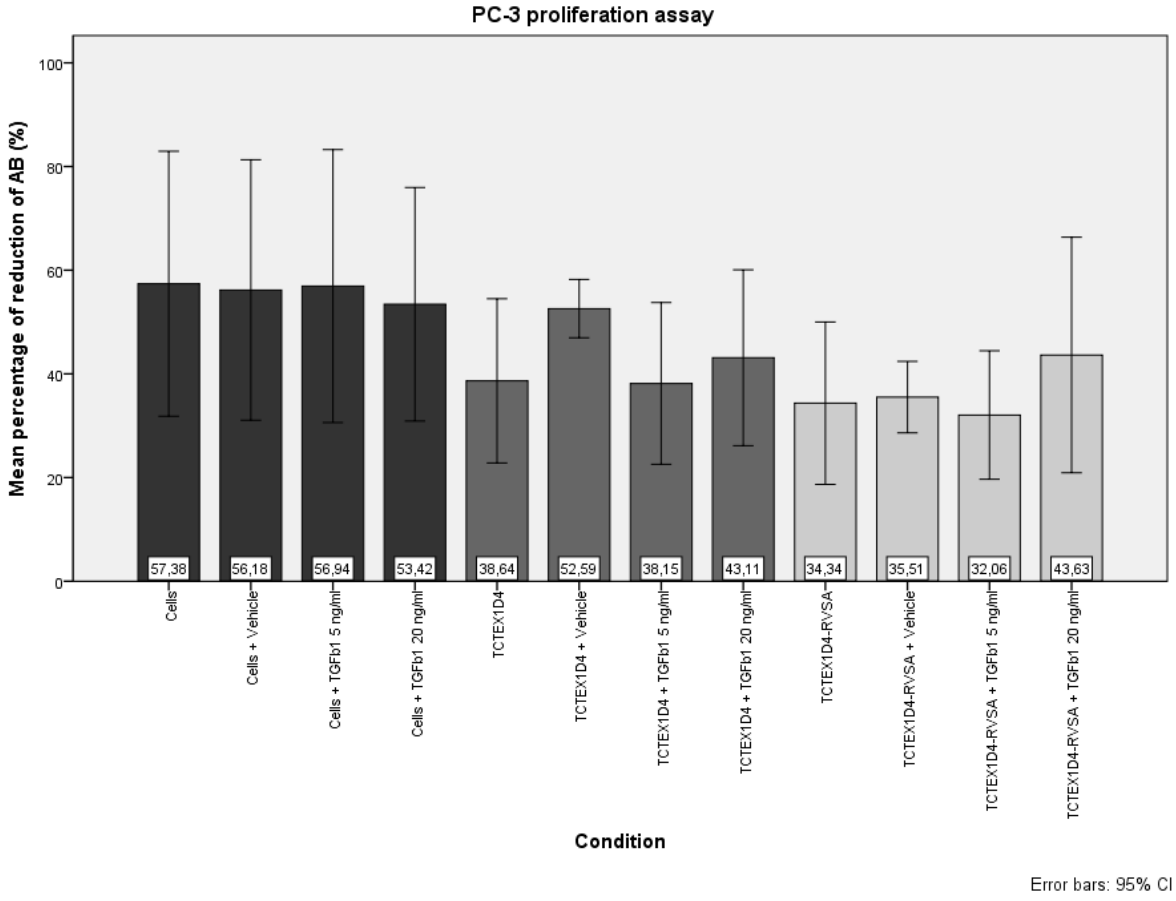


Figure 14 | Proliferation of PC-3 transfected cells in response to TGFβ1.

Cells were treated with TGFβ1 (5 ng/ml or 20 ng/ml), left untreated or treated with the vehicle. The graph shows the average percentage of reduction of AB. Data represent the mean and 95% confidence intervals (95% CI) for three independent experiments, each of three replicates for each condition. Mean value is displayed for each condition. Mean values ± Standard Deviation were as follows: Cells: 57.38±33.23%; cells + vehicle: 56.18±32.68; cells + TGFβ1 5 ng/ml: 56.94±34.25; cells + TGFβ1 20 ng/ml: 53.42±29.31%; TCTEX1D4: 38.64±20.60; TCTEX1D4 + vehicle: 52.59±5.38; TCTEX1D4 TGFβ1 5 ng/ml: 38.15±20.29; TCTEX1D4 TGFβ1 20 ng/ml: 43.11±20.30; TCTEX1D4-RVSA: 34.34±20.37; TCTEX1D4-RVSA + vehicle: 35.51±6.59 ;TCTEX1D4-RVSA TGFβ1 5 ng/ml: 32.06±16.12; TCTEX1D4-RVSA TGFβ1 20 ng/ml: 43.63±29.57. Differences in the variable "transfected protein" were statistically significant ($p < 0.001$).

4.5.4 ADDITIONAL ANALYSIS OF STATISTICAL SIGNIFICANT DIFFERENCES

The results from the Kruskal-Wallis test state that there are differences between the median proliferation rates among non-transfected cells, TCTEX1D4 transfected cells, and TCTEX1D4-RVSA transfected cells populations. To further analyze these differences, we

performed the Mann-Whitney U test with Bonferroni correction (α=0.017) in order to conduct multiple comparisons between groups.

The results show that for PNT-2 cells only the comparison between TCTEX1D4 and TCTEX1D4-RVSA populations reached statistical significance (p=0.004). Thus, the proliferation rate of PNT-2 cells when the ability to binding to PPP1 is decreased is significantly lower than when cells express wild type TCTEX1D4.

For PC-3 cells, all multiple comparisons performed show statistical significance (non-transfected cells versus TCTEX1D4 transfected cells: p=0.001; non-transfected cells versus TCTEX1D4-RVSA transfected cells: p=0.002; and TCTEX1D4 transfected cells versus TCTEX1D4-RVSA transfected cells: p=0.007). Hence, it appears that cells exhibit a lower proliferation rate when express wild type TCTEX1D4, but this decrease is even more pronounced when TCTEX1D4 is mutated in the RVSF motif, compromising its binding to PPP1.

4.6 MIGRATION OF TRANSFECTED CELLS IN RESPONSE TO TGFβ1

To unravel the influence of TCTEX1D4/PPP1 complex in cell migration, human prostate cells were transfected with the plasmids Myc-TCTEX1D4 and Myc-TCTEX1D4-RVSA for the reasons already mentioned in proliferation. After that, cells were seeded in the seeder plate of the HTS Transwell Permeable Support and allowed to migrate for 24 hours through a microporous membrane in response to low (5 ng/ml) or high (20 ng/ml) concentrations of TGFβ1.

The number of migratory cells in the reservoir plate was then assessed using AB and the percentage of reduction of AB was calculated according to Figure 10 [76].

4.6.1 PNT-2

Cells transfected with TCTEX1D4-RVSA showed a slight improvement in their migratory potential (18.53%) when compared to control cells (14.90%) or cells transfected with TCTEX1D4 (16.00%), as depicted in Figure 15.

The addition of higher concentrations of TGFβ1 to the experimental environment generated a tiny decrease in the migration of PNT-2 (from 15.12% to 12.44%). In TCTEX1D4, TGFβ1 decreased the cell migratory behavior in a dose-dependent manner

(from 18.77% to 16.84% and 15.87%). On the other hand, higher concentrations of TGFβ1 potentiate the migratory potential of TCTEX1D4 transfected cells (22.53% compared with 17.43% of the vehicle control). However, any of the differences were statistically significant.

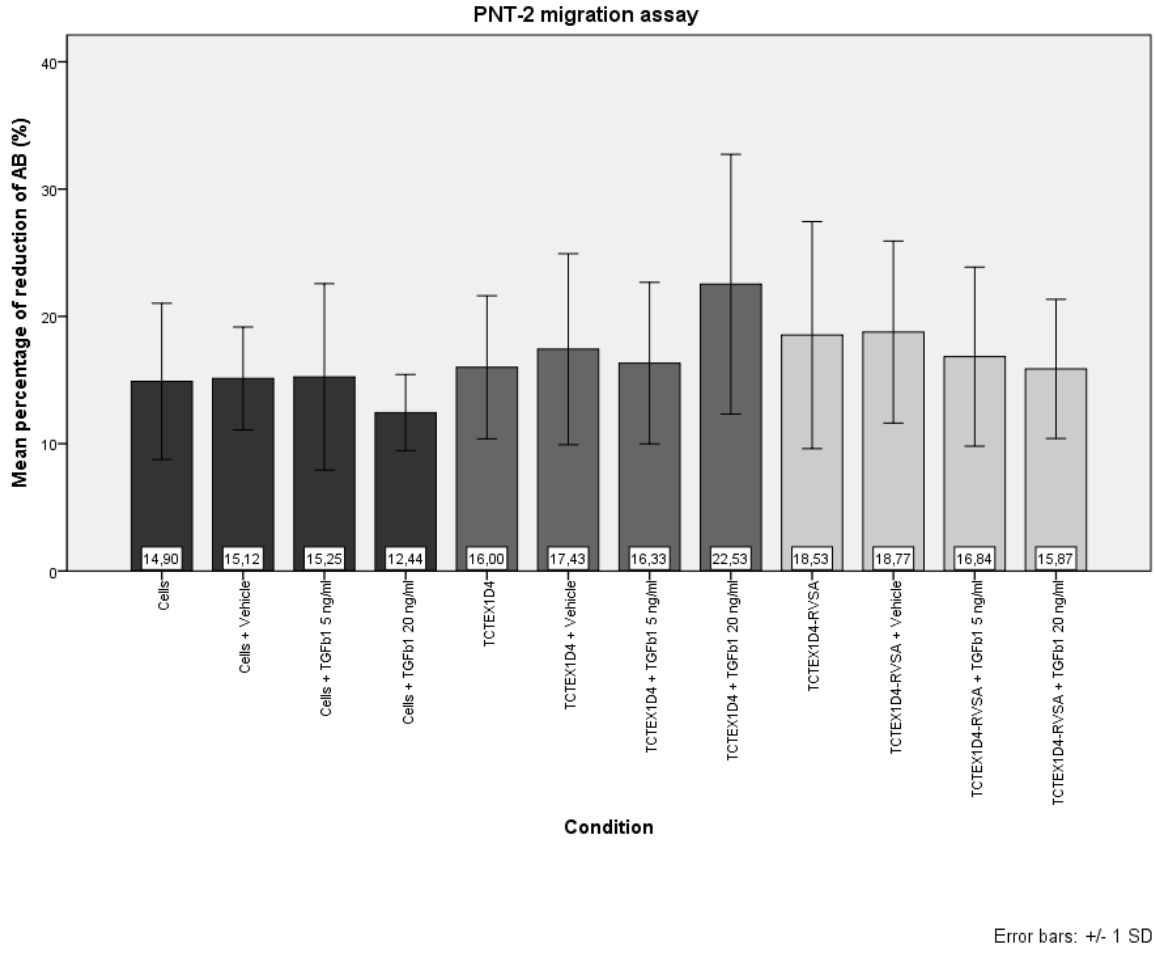


Figure 15 | Migration of PNT-2 transfected cells in response to TGFβ1.

Cells were treated with TGFβ1 (5 ng/ml or 20 ng/ml), left untreated or treated with the vehicle for 24 hours. The graph shows the average percentage of reduction of AB. Data represent the mean ± SD for two independent experiments, each of two replicates for each condition. Mean value is displayed for each condition. Mean values ± Standard Deviation were as follows: Cells: 14.90±6.13%; cells + vehicle: 15.12±4.04; cells + TGFβ1 5 ng/ml: 15.25±7.32; cells + TGFβ1 20 ng/ml: 12.44±2.99%; TCTEX1D4: 16.00±5.22; TCTEX1D4 + vehicle: 17.43±7.50; TCTEX1D4 TGFβ1 5 ng/ml: 16.33±6.34; TCTEX1D4 TGFβ1 20 ng/ml: 22.53±10.20; TCTEX1D4-RVSA: 18.53±8.92; TCTEX1D4-RVSA + vehicle: 7.15±13.13 ;TCTEX1D4-RVSA TGFβ1 5 ng/ml: 16.84±7.03; TCTEX1D4-RVSA TGFβ1 20 ng/ml: 15.87±5.46.

4.6.2 LNCaP

As can be observed in Figure 16, there is virtually no difference among the migratory behavior of LNCaP cells (17.05%), TCTEX1D4 transfected cells (18.76%), and TCTEX1D4-RVSA cells (18.43%).

The addition of TGFβ1 appears to reduce the migration rate in all conditions, but differences were not significant.

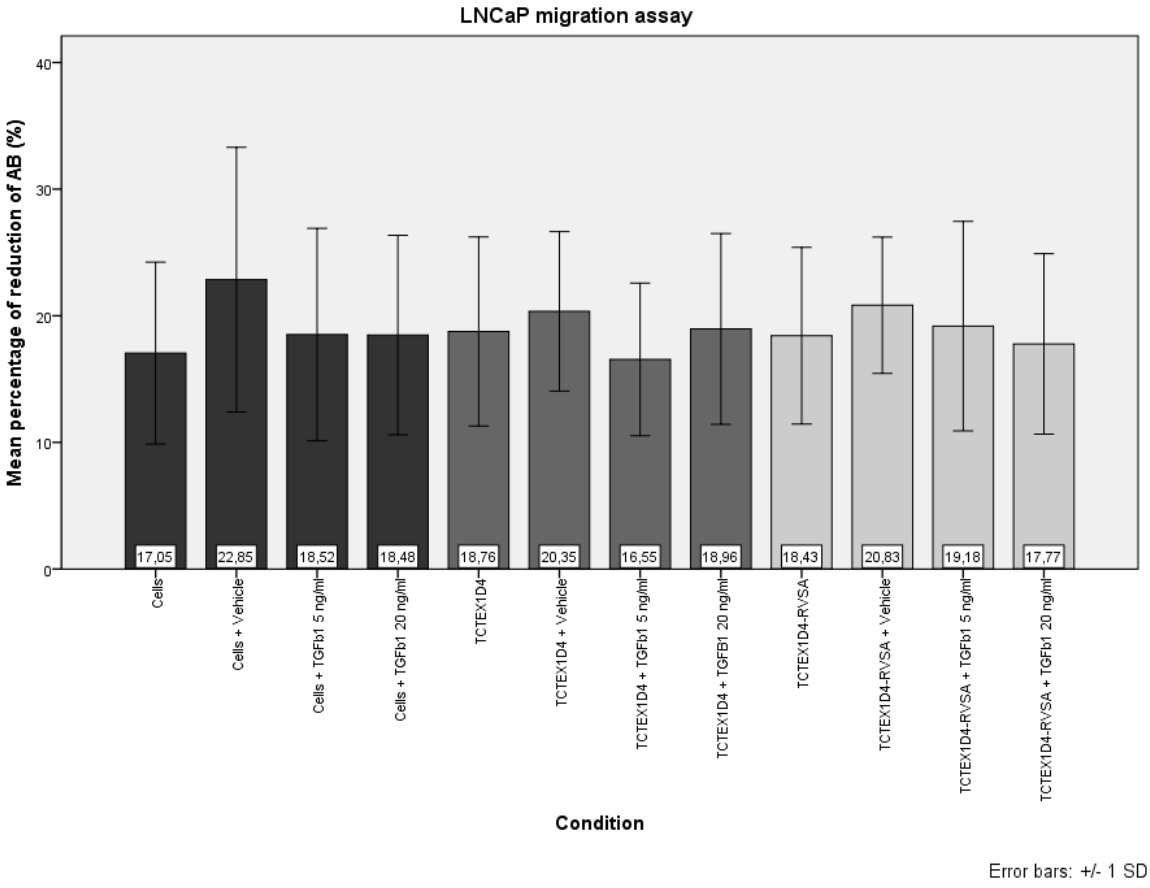


Figure 16 | Migration of LNCaP transfected cells in response to TGFβ1.

Cells were treated with TGFβ1 (5 ng/ml or 20 ng/ml), left untreated or treated with the vehicle for 24 hours. The graph shows the average percentage of reduction of AB. Data represent the mean ± SD for two independent experiments, each of two replicates for each condition. Mean value is displayed for each condition. Mean values ± Standard Deviation were as follows: Cells: 17.05±7.19%; cells + vehicle: 22.85±10.46; cells + TGFβ1 5 ng/ml: 18.52±8.39; cells + TGFβ1 20 ng/ml: 18.48±7.87%; TCTEX1D4: 18.76±7.46; TCTEX1D4 + vehicle: 20.35±6.30; TCTEX1D4 TGFβ1 5 ng/ml: 16.55±6.03; TCTEX1D4 TGFβ1 20 ng/ml: 18.96±7.54; TCTEX1D4-RVSA: 18.43±6.98; TCTEX1D4-RVSA + vehicle: 20.83±5.39 ;TCTEX1D4-RVSA TGFβ1 5 ng/ml: 19.18±8.27; TCTEX1D4-RVSA TGFβ1 20 ng/ml: 17.77±7.13.

4.6.3 PC-3

As depicted in Figure 17, the transfection with TCTEX1D4 or its mutant did not produce any alteration in cell migration rate.

The addition of TGFβ1 resulted in a decrease in migration in both non-transfected cells and cells transfected with TCTEX1D4. In TCTEX1D4-RVSA transfected cells, the addition of TGFβ1 did not produced any alteration in the migration rates.

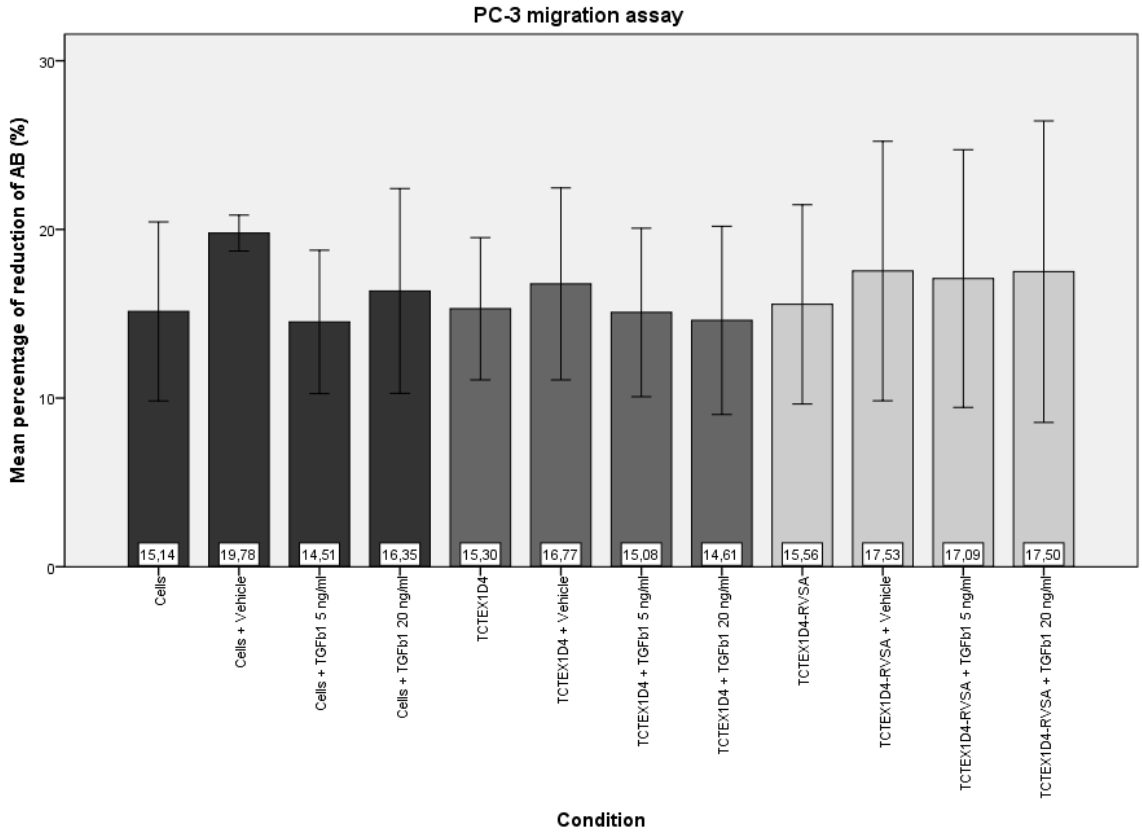


Figure 17 | Migration of PC-3 transfected cells in response to TGFβ1.

Cells were treated with TGFβ1 (5 ng/ml or 20 ng/ml), left untreated or treated with the vehicle for 24 hours. The graph shows the average percentage of reduction of AB. Data represent the mean ± SD for two independent experiments, each of two replicates for each condition. Mean value is displayed for each condition. Mean values ± Standard Deviation were as follows: Cells: 15.14±5.30%; cells + vehicle: 19.78±1.07; cells + TGFβ1 5 ng/ml: 14.51±4.25; cells + TGFβ1 20 ng/ml: 16.35±6.07%; TCTEX1D4: 15.30±4.22; TCTEX1D4 + vehicle: 16.77±5.69; TCTEX1D4 TGFβ1 5 ng/ml: 15.08±5.00; TCTEX1D4 TGFβ1 20 ng/ml: 14.61±5.58; TCTEX1D4-RVSA: 15.56±5.91; TCTEX1D4-RVSA + vehicle: 17.53±7.69 ;TCTEX1D4-RVSA TGFβ1 5 ng/ml: 17.09±7.64; TCTEX1D4-RVSA TGFβ1 20 ng/ml: 17.50±8.94.

DISCUSSION

In the present study we aimed to unravel the effect of TCTEX1D4 and the role of its binding to PPP1 in cell proliferation and migration. We took advantage of two plasmids [70], one expressing wild type TCTEX1D4 and the other expressing a mutant form in the PPP1 binding motif from RVSF to RVSA. This mutant TCTEX1D4 binds to PPP1 with a 35% less efficiency.

To achieve our main goal, we firstly performed several experiments in order to optimize cell transfection method with Lipofectamine 2000. With the selected conditions, we were able to achieve transfection efficiencies above 30% for all cell lines, which is already suitable to respond our main goals.

As TCTEX1D4 was found to interfere with TGFβ signaling pathway, we stimulated cells with TGFβ1 in order to achieve a broader comprehension on TCTEX1D4 function. TGFβ1 is a cytokine known to inhibit proliferation of epithelial cells. Actually, it has been recognized as a challenging barrier to the development of cancer hallmarks, as it inhibits cell proliferation, migration and invasion, while promotes apoptosis, cell adhesion, and cellular differentiation. However, in late-stage tumors, the cellular machinery subverts the TGFβ signaling pathway in order to promote cancer progression.

In normal prostate cells, as well as in prostate cancer cells, TGFβ effects are, nevertheless, not well-established and some questions still in debate. PNT-2 cells have been described as being responsive to TGFβ1, and their proliferation was shown to be inhibited by its signal [72]. PC-3 cells have been referred as being partially responsive to the inhibitory effects of TGFβ1 due to the loss of TGFβ receptors with PCa progression [77]. LNCaP cells are the more debated ones; some authors defend that they are totally resistant to the anti-proliferative effects of TGFβ1 and exhibit a complete absence of TβRI mRNA transcripts [77], whereas others demonstrated that they could be responsive in certain conditions [78]. Also, the differences in human prostate cells response to TGFβ1 appear to be extremely related to the TGFβ1 concentration used.

In this study, using 5 ng/ml and 20 ng/ml of TGFβ1, we verified that: (1) in PNT-2 cells, 5 ng/ml of TGFβ1 decreased cells proliferation as previously described [72], but cell proliferation returned to approximately normal levels when cells were exposed to 20 ng/ml TGFβ1; (2) LNCaP were responsive, presenting a decrease in proliferation in response to both concentrations of TGFβ1; (3) PC-3 were virtually non-responsive. In terms of migratory behavior, all the three cell lines showed a decrease in migration in response to TGFβ1, although this effect in PNT-2 was only observed with the highest concentration.

However, these results are difficult to compare due to the variability associated to them. The presence of large error bars could be partially explained by differences in the transfection efficiency between independent experiments. Also, AB is sensible for as less as 50 cells. Thus, even small inconsistencies in cell counting, resuspension and consequent plating can result in slightly distinct absorbance measurements. That is way it is recommended to perform replicates of each measurement. So, to be sure of the effects of TGFβ1 in the cell lines used we should increase the number of measurements for each condition in order to decrease the associated error.

In spite of the variability associated with our data, we obtained statistically significant data that allowed us to progress on the understanding of TCTEX1D4 and to understand the importance of its regulation via binding to PPP1.

TCTEX1D4 as an anti-proliferative agent

TCTEX1D4 is a dynein light chain protein whose function remains poorly understood. It has been proposed to be a player in the TGFβ signaling pathway. More precisely,

TCTEX1D4 binds to two TGFβ receptors, TβRII and endoglin, and retained them in the cellular membrane [67]. By this way, receptors cannot be internalized and the process of receptors recycling is compromised. This virtually culminates in the inhibition of Smad2/3, whose signal is known to lead to the acquisition of malignant capabilities in late-stage tumors, including invasiveness, migration, and metastasis formation [38, 39]. So, TCTEX1D4 is expected to act as a tumor suppressor by inhibiting Smad2/3 signaling. In fact, the results obtained corroborate this hypothesis as TCTEX1D4 decreased cell proliferation in the three cell lines used, with the results being statistically significant for PNT-2 and PC-3 cells.

TGFβ1 addition decreased cell proliferation in cells transfected with TCTEX1D4 and TCTEX1D4-RVSA in almost all cell lines when compared to the control vehicle (the exception is a high concentration of TGFβ1 in PC-3 transfected with TCTEX1D4-RVSA, where proliferation seemed to be enhanced). However, these alterations were not significant. Taking into account the current theoretical knowledge about TCTEX1D4, if the more prominent mode of action of TCTEX1D4 in TGFβ signaling is achieved by the maintenance of TβRII receptors in the cell membrane, it is likely that they virtually become overcrowded and no more sites are available for ligand binding. Therefore, it is expected that the addition of TGFβ1 in TCTEX1D4 transfected cells produce limited anti-proliferative effects.

PPP1 acts as a 'switch off' of TCTEX1D4 activity

Extensive bioinformatics analysis of TCTEX1D4 structure performed in our laboratory demonstrated that TCTEX1D4 presents many putative serine phosphorylation sites while no threonine or tyrosine phospho-sites were found. This indicates that serine phosphorylation might be a primarily mechanism for its regulation. PPP1 is a major phosphatase specific for serine and threonine residues, and accordingly, it was found the RVSF motif in the N-terminus of TCTEX1D4 [70]. Thus, this evidence suggests that TCTEX1D4 function might be regulated via dephosphorylation by PPP1.

The mutation in one amino acid of the RVSF motif of TCTEX1D4 was previously shown to decrease the bind of TCTEX1D4 to PPP1 in about 35% [70]. Hence, TCTEX1D4-RVSA transfected cells mimic a state where there is an impairment of the formation of TCTEX1D4/PPP1 complex and, consequently, a putatively weakening in TCTEX1D4

dephosphorylation. The results indicate that cells transfected with the TCTEX1D4 mutant (TCTEX1D4-RVSA) showed an even more pronounced decrease in cell proliferation than TCTEX1D4 transfected cells in all cell lines. So, it appears that when PPP1 binding to TCTEX1D4 is compromised and dephosphorylation of TCTEX1D4 may occurred in a less extended manner, the inhibitory effects of TCTEX1D4 in cell proliferation are promoted. Therefore, PPP1 dephosphorylation of TCTEX1D4 might constitute a 'switch off' of TCTEX1D4 function, contributing to cell proliferation.

In accordance to the above mentioned idea in what concerns TGFβ1 effect in TCTEX1D4 transfected cells, TGFβ1 effect in TCTEX1D4-RVSA should be even more limited as TCTEX1D4 activity is augmented. Instead, our results still demonstrate a decrease in cell proliferation ratio in TCTEX1D4-RVSA transfected cells, as already mentioned, with the only exception being PC-3 TCTEX1D4-RVSA transfected cells. Once again, any of these alterations were statistically significant.

TCTEX1D4 as a regulator of PPP1 subcellular localization

An alternative or complementary explanation for TCTEX1D4/PPP1 complex functionality would be the targeting of PPP1 by TCTEX1D4. PPP1C has been already shown to be a noticeable regulator of several membrane receptors. For instance, in TGFβ signaling, PPP1C is presented to ALK5 receptor by the docking protein SARA, a targeting also involves GADD34 and Smad7. As a result, ALK5 is dephosphorylated and there is an attenuation of ALK5-mediating signaling. Also, PPP1C is recruited by Smad7 to ALK1, inhibiting the signaling via Smad1/5/8.

As TCTEX1D4 is a dynein light chain protein, responsible for cargo binding, it is likely to bind PPP1 and modulate its subcellular localization. Dynein motor complexes are responsible for retrograde transport so, TCTEX1D4 might be responsible for capture PPP1 in the cell membrane and transport it to intracellular compartments. Once there, PPP1 might dephosphorylate key molecules for PCa progression. In fact, it was already demonstrated that PPP1 directly dephosphorylates and attenuates two major tumor suppressors, p53 and pRb, whose deregulation has been associated with PCa [52, 79, 80].

As so, it might be expected that the disruption of the complex formation between TCTEX1D4 and PPP1 compromised the transport of PPP1 from the periphery to more

central localizations within the cell. Hence, TCTEX1D4 may indirectly regulate PPP1 function by determining its subcellular localization.

TCTEX1D4 role in cell migration is still cloudy

In contrast to proliferation, where TCTEX1D4 seems to assume an important function as regulator, in migration its effects appear to be almost insignificant with only minor differences being observed. However, we are not able to discard a role for TCTEX1D4 and also for the complex that it forms with PPP1 in cell migration, as the incubation time used may not be sufficient to produce any significant migration. Moreover, the lack of differences could be due to the high data variability observed. The number of measurements and independent experiments is still lower (two replicates for each sample of two independent experiments), which is translated in large error bars that make difficult to establish comparisons among conditions.

For these reasons, migratory potential of TCTEX1D4 and TCTEX1D4-RVSA transfected cells should be assessed using other methods that could provide a real-time analysis of cell movement.

CONCLUSIONS

The study of cell signaling pathways and respective alterations in pathological processes has been receiving increasing attention as it enables the identification of molecules that may constitute potential biomarkers for diseases and/or new therapeutical targets [18].

The particular relevance of such study in PCa is not only due to the high incidence and prevalence levels that it currently assumes (and that tend to increase along with population aging), but also because it is a clinically silent disease, particularly in early stages [81]. Another problem in PCa management started with the widespread implementation PSA screening, which led to the diagnosis and treatment of several PCa cases that would not otherwise cause symptoms or threaten man's life. Altogether, these problems determine a particular urgency in the establishment of a specific panel of biomarkers in body fluids that empowered the diagnosis and follow-up of patients, and the discovery of new therapeutic targets [18].

Evidences suggest that modulation of TGFβ signaling aid the control of tumor progression. Thus, several mediators of TGFβ signaling pathway have been regarded as interesting targets for PCa therapy. Actually, genistein, a compound that is already ongoing phase II of clinical trials, acts via activation of Smad1, in an ALK2-dependent way, thus suppressing PCa invasiveness [77]. On the other hand, efforts are now being made by

pharmaceutical companies to investigate the role of phosphoprotein phosphatases as therapeutical targets. However, because of the numerous functions of PPP1 catalytic subunit, the long-term usage of these enzyme inhibitors has been associated with nephrotoxicity and hepatotoxicity. For this reason, the adequate option seems to be targeting PIPs instead of protein phosphatases directly as they are more event, tissue and subcellular compartment specific [44].

In this study we demonstrated that a recently described PIP, TCTEX1D4, is an anti-proliferative agent, as it decreased human prostate cells proliferation. Moreover, we showed that the impairment of the complex formation between TCTEX1D4 and PPP1 culminates in a more exacerbate anti-proliferative activity of TCTEX1D4. Therefore, PPP1 may be an inhibitory regulator of TCTEX1D4 anti-proliferative actions. Alternatively, or in a complementary manner, TCTEX1D4 may indirectly regulate PPP1 function through modification of its subcellular localization.

For this reasons, TCTEX1D4/PPP1 complex might constitute a new therapeutical target for PCa, as it is expected that its disruption by small molecules (e.g. peptides [47]) leads to a decrease of PCa cell proliferation.

To further explore this hypothesis, studies with mutants that mimic the phosphorylation state of TCTEX1D4 and short hairpin ribonucleic acids (shRNAs) for TCTEX1D4 are already being performed in the laboratory.

REFERENCES

1. Hall, J.E. Guyton and Hall Textbook of Medical Physiology: Saunders; 2010.
2. Kumar, V.L., Majumder, P.K. Prostate gland: structure, functions and regulation. International urology and nephrology. 1995;27(3):231-43. Epub 1995/01/01.
3. Ross, M.H., Pawlina, W. Histology: A Text and Atlas: Lippincott Williams & Wilkins; 2010.
4. Stevens, A., Lowe, J. Human Histology: Mosby; 2004.
5. Lee, C.H., Akin-Olugbade, O., Kirschenbaum, A. Overview of prostate anatomy, histology, and pathology. Endocrinology and metabolism clinics of North America. 2011;40(3):565-75, viii-ix. Epub 2011/09/06.
6. VanPutte, C., Regan, J., Russo, A. Seeley's Anatomy & Physiology: McGraw-Hill Science; 2010.
7. Ross, M.H., Pawlina, W. Histology: A Text and Atlas. 6th ed: Lippincott Williams & Wilkins; 2010.
8. Konishi, N., Shimada, K., Ishida, E., Nakamura, M. Molecular pathology of prostate cancer. Pathology international. 2005;55(9):531-9. Epub 2005/09/07.
9. Long, R.M., Morrissey, C., Fitzpatrick, J.M., Watson, R.W. Prostate epithelial cell differentiation and its relevance to the understanding of prostate cancer therapies. Clin Sci (Lond). 2005;108(1):1-11. Epub 2004/09/24.
10. Lonergan, P.E., Tindall, D.J. Androgen receptor signaling in prostate cancer development and progression. Journal of carcinogenesis. 2011;10:20. Epub 2011/09/03.
11. International Agency for Research on Cancer. Globocan 2008. World Health Organization; 2008; Available from: http://globocan.iarc.fr/.
12. Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C., Parkin, D.M. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International journal of cancer Journal international du cancer. 2010;127(12):2893-917. Epub 2011/02/26.
13. Nelson, W.G., De Marzo, A.M., Isaacs, W.B. Prostate cancer. The New England journal of medicine. 2003;349(4):366-81. Epub 2003/07/25.
14. Crawford, E.D. Understanding the epidemiology, natural history, and key pathways involved in prostate cancer. Urology. 2009;73:S4-10. Epub 2009/04/23.

15. Woude, G.F.V., Klein, G. Advances in Cancer Research: Academic Press; 2010.
16. Lu, T.L., Huang, Y.F., You, L.R., Chao, N.C., Su, F.Y., Chang, J.L., Chen, C.M. Conditionally Ablated Pten in Prostate Basal Cells Promotes Basal-to-Luminal Differentiation and Causes Invasive Prostate Cancer in Mice. The American journal of pathology. 2013;182(3):975-91. Epub 2013/01/15.
17. Dasgupta, S., Srinidhi, S., Vishwanatha, J.K. Oncogenic activation in prostate cancer progression and metastasis: Molecular insights and future challenges. Journal of carcinogenesis. 2012;11:4. Epub 2012/03/23.
18. Felgueiras, J., Silva, J.V., Fardilha, M. Prostate cancer: the need for biomarkers and new therapeutic targets. Journal of Zhejiang University SCIENCE B. 2013. [Accepted on 2013/06/08]
19. Bubendorf, L., Schopfer, A., Wagner, U., Sauter, G., Moch, H., Willi, N., Gasser, T.C., Mihatsch, M.J. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Human pathology. 2000;31(5):578-83. Epub 2000/06/03.
20. Clarke, N.W., Hart, C.A., Brown, M.D. Molecular mechanisms of metastasis in prostate cancer. Asian journal of andrology. 2009;11(1):57-67. Epub 2008/12/04.
21. Ikushima, H., Miyazono, K. Biology of transforming growth factor-beta signaling. Current pharmaceutical biotechnology. 2011;12(12):2099-107. Epub 2011/05/31.
22. Massague, J. TGFbeta signalling in context. Nature reviews Molecular cell biology. 2012;13(10):616-30. Epub 2012/09/21.
23. Korrodi-Gregório, L., Teixeira, A.L., Medeiros, R. Sinalização via TGFβ. In: Afrontamento, E., editor. O eSsencial em Sinalização Celular2012a.
24. Kubiczkova, L., Sedlarikova, L., Hajek, R., Sevcikova, S. TGF-beta - an excellent servant but a bad master. Journal of translational medicine. 2012;10:183. Epub 2012/09/05.
25. Elliott, R.L., Blobe, G.C. Role of transforming growth factor Beta in human cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23(9):2078-93. Epub 2005/03/19.
26. Moustakas, A., Heldin, C.H. The regulation of TGF beta signal transduction. Development. 2009;136(22):3699-714.
27. Santibanez, J.F., Quintanilla, M., Bernabeu, C. TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. Clin Sci (Lond). 2011;121(6):233-51. Epub 2011/05/28.
28. Inoue, Y., Imamura, T. Regulation of TGF-beta family signaling by E3 ubiquitin ligases. Cancer science. 2008;99(11):2107-12. Epub 2008/09/24.
29. Massague, J., Seoane, J., Wotton, D. Smad transcription factors. Genes & development. 2005;19(23):2783-810. Epub 2005/12/03.
30. Bello-DeOcampo, D., Tindall, D.J. TGF-betal/Smad signaling in prostate cancer. Current drug targets. 2003;4(3):197-207. Epub 2003/03/20.
31. Wikstrom, P., Stattin, P., Franck-Lissbrant, I., Damber, J.E., Bergh, A. Transforming growth factor beta1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. The Prostate. 1998;37(1):19-29. Epub 1998/08/28.
32. Matthews, E., Yang, T., Janulis, L., Goodwin, S., Kundu, S.D., Karpus, W.J., Lee, C. Down-regulation of TGF-beta1 production restores immunogenicity in prostate cancer cells. British journal of cancer. 2000;83(4):519-25. Epub 2000/08/17.
33. Guo, Y.P., Jacobs, S.C., Kyprianou, N. Down-regulation of protein and mRNA expression for transforming growth factor-beta (TGF-beta 1) type I and type II receptors in human prostate cancer. International Journal of Cancer. 1997;71(4):573-9.
34. Blanchere, M., Saunier, E., Mestayer, C., Broshuis, M., Mowszowicz, I. Alterations of expression and regulation of transforming growth factor beta in human cancer prostate cell lines. The Journal of steroid biochemistry and molecular biology. 2002;82(4-5):297-304. Epub 2003/02/19.
35. Guo, Y., Kyprianou, N. Restoration of transforming growth factor beta signaling pathway in human prostate cancer cells suppresses tumorigenicity via induction of caspase-1-mediated apoptosis. Cancer research. 1999;59(6):1366-71. Epub 1999/03/30.

36. Gatza, C.E., Oh, S.Y., Blobe, G.C. Roles for the type III TGF-beta receptor in human cancer. Cell Signal. 2010;22(8):1163-74. Epub 2010/02/16.
37. Craft, C.S., Romero, D., Vary, C.P., Bergan, R.C. Endoglin inhibits prostate cancer motility via activation of the ALK2-Smad1 pathway. Oncogene. 2007;26(51):7240-50. Epub 2007/05/15.
38. Romero, D., O'Neill, C., Terzic, A., Contois, L., Young, K., Conley, B.A., Bergan, R.C., Brooks, P.C., Vary, C.P.H. Endoglin Regulates Cancer-Stromal Cell Interactions in Prostate Tumors. Cancer research. 2011;71(10):3482-93.
39. Romero, D., Terzic, A., Conley, B.A., Craft, C.S., Jovanovic, B., Bergan, R.C., Vary, C.P.H. Endoglin phosphorylation by ALK2 contributes to the regulation of prostate cancer cell migration. Carcinogenesis. 2010;31(3):359-66.
40. Kirkbride, K.C., Blobe, G.C. Inhibiting the TGF-beta signalling pathway as a means of cancer immunotherapy. Expert Opin Biol Th. 2003;3(2):251-61.
41. Jones, E., Pu, H., Kyprianou, N. Targeting TGF-beta in prostate cancer: therapeutic possibilities during tumor progression. Expert opinion on therapeutic targets. 2009;13(2):227-34. Epub 2009/02/25.
42. Lakshman, M., Huang, X., Ananthanarayanan, V., Jovanovic, B., Liu, Y., Craft, C.S., Romero, D., Vary, C.P., Bergan, R.C. Endoglin suppresses human prostate cancer metastasis. Clinical & experimental metastasis. 2011;28(1):39-53. Epub 2010/10/29.
43. Rocha, J., Ferreira, M., Fardilha, M. Fosforilação de proteínas na sinalização celular. In: Afrontamento, E., editor. O eSsencial em Sinalização Celular2012.
44. Fardilha, M., Esteves, S.L., Korrodi-Gregorio, L., da Cruz e Silva, O.A., da Cruz e Silva, F.F. The physiological relevance of protein phosphatase 1 and its interacting proteins to health and disease. Current medicinal chemistry. 2010;17(33):3996-4017. Epub 2010/10/14.
45. Shi, Y. Serine/Threonine Phosphatases: Mechanism through Structure. Cell. 2009;139(3):468-84.
46. Graves, J.D., Krebs, E.G. Protein phosphorylation and signal transduction. Pharmacol Therapeut. 1999;82(2-3):111-21.
47. Cohen, P.T.W. Protein phosphatase 1 - targeted in many directions. Journal of cell science. 2002;115(2):241-56.
48. Ceulemans, H., Bollen, M. Functional diversity of protein phosphatase-1, a cellular economizer and reset button. Physiological reviews. 2004;84(1):1-39. Epub 2004/01/13.
49. Bollen, M., Peti, W., Ragusa, M.J., Beullens, M. The extended PP1 toolkit: designed to create specificity. Trends in biochemical sciences. 2010;35(8):450-8. Epub 2010/04/20.
50. Kuntziger, T., Landsverk, H.B., Collas, P., Syljuasen, R.G. Protein phosphatase 1 regulators in DNA damage signaling. Cell cycle (Georgetown, Tex). 2011;10(9):1356-62. Epub 2011/04/01.
51. Li, D.W., Liu, J.P., Schmid, P.C., Schlosser, R., Feng, H., Liu, W.B., Yan, Q., Gong, L., Sun, S.M., Deng, M., *et al.* Protein serine/threonine phosphatase-1 dephosphorylates p53 at Ser-15 and Ser-37 to modulate its transcriptional and apoptotic activities. Oncogene. 2006;25(21):3006-22. Epub 2006/02/28.
52. Amatangelo, M.D., Goodyear, S., Varma, D., Stearns, M.E. c-Myc expression and MEK1-induced Erk2 nuclear localization are required for TGF-beta induced epithelial-mesenchymal transition and invasion in prostate cancer. Carcinogenesis. 2012;33(10):1965-75. Epub 2012/07/14.
53. Allard, P., Zoubeidi, A., Nguyen, L.T., Tessier, S., Tanguay, S., Chevrette, M., Aprikian, A., Chevalier, S. Links between Fer tyrosine kinase expression levels and prostate cell proliferation. Mol Cell Endocrinol. 2000;159(1-2):63-77. Epub 2000/02/25.
54. Kaelin, W.G., Jr. Functions of the retinoblastoma protein. BioEssays : news and reviews in molecular, cellular and developmental biology. 1999;21(11):950-8. Epub 1999/10/12.
55. Pasder, O., Shpungin, S., Salem, Y., Makovsky, A., Vilchick, S., Michaeli, S., Malovani, H., Nir, U. Downregulation of Fer induces PP1 activation and cell-cycle arrest in malignant cells. Oncogene. 2006;25(30):4194-206. Epub 2006/05/30.
56. Karam, J.A., Lotan, Y., Roehrborn, C.G., Ashfaq, R., Karakiewicz, P.I., Shariat, S.F. Caveolin-1 overexpression is associated with aggressive prostate cancer recurrence. The Prostate. 2007;67(6):614-22. Epub 2007/02/15.

57. Li, L., Ren, C.H., Tahir, S.A., Ren, C., Thompson, T.C. Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Molecular and cellular biology. 2003;23(24):9389-404. Epub 2003/12/04.

58. Chen, S., Kesler, C.T., Paschal, B.M., Balk, S.P. Androgen receptor phosphorylation and activity are regulated by an association with protein phosphatase 1. The Journal of biological chemistry. 2009;284(38):25576-84. Epub 2009/07/23.

59. Martin-Granados, C., Prescott, A.R., Van Dessel, N., Van Eynde, A., Arocena, M., Klaska, I.P., Gornemann, J., Beullens, M., Bollen, M., Forrester, J.V., *et al.* A role for PP1/NIPP1 in steering migration of human cancer cells. PloS one. 2012;7(7):e40769. Epub 2012/07/21.

60. McConnell, J.L., Wadzinski, B.E. Targeting protein serine/threonine phosphatases for drug development. Mol Pharmacol. 2009;75(6):1249-61. Epub 2009/03/21.

61. Bennett, D., Alphey, L. PP1 binds Sara and negatively regulates Dpp signaling in Drosophila melanogaster. Nature genetics. 2002;31(4):419-23. Epub 2002/07/23.

62. Shi, W., Sun, C., He, B., Xiong, W., Shi, X., Yao, D., Cao, X. GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. The Journal of cell biology. 2004;164(2):291-300. Epub 2004/01/14.

63. Valdimarsdottir, G., Goumans, M.J., Itoh, F., Itoh, S., Heldin, C.H., ten Dijke, P. Smad7 and protein phosphatase 1alpha are critical determinants in the duration of TGF-beta/ALK1 signaling in endothelial cells. BMC cell biology. 2006;7:16. Epub 2006/03/31.

64. Korrodi-Gregório, L. Characterization of PP1 interacting proteins in male reproduction: University of Aveiro; 2012b.

65. King, S.M. The dynein microtubule motor. Biochimica et biophysica acta. 2000;1496(1):60-75. Epub 2000/03/21.

66. Karcher, R.L., Deacon, S.W., Gelfand, V.I. Motor-cargo interactions: the key to transport specificity. Trends in cell biology. 2002;12(1):21-7. Epub 2002/02/21.

67. Meng, Q., Lux, A., Holloschi, A., Li, J., Hughes, J.M., Foerg, T., McCarthy, J.E., Heagerty, A.M., Kioschis, P., Hafner, M., *et al.* Identification of Tctex2beta, a novel dynein light chain family member that interacts with different transforming growth factor-beta receptors. The Journal of biological chemistry. 2006;281(48):37069-80. Epub 2006/09/20.

68. Hook, P., Vallee, R.B. The dynein family at a glance. Journal of cell science. 2006;119(Pt 21):4369-71. Epub 2006/11/01.

69. DiBella, L.M., Benashski, S.E., Tedford, H.W., Harrison, A., Patel-King, R.S., King, S.M. The Tctex1/Tctex2 class of dynein light chains. Dimerization, differential expression, and interaction with the LC8 protein family. The Journal of biological chemistry. 2001;276(17):14366-73. Epub 2001/03/30.

70. Korrodi-Gregório, L., Vieira, S.I., Esteves, S.L.C., Silva, J.V., Freitas, M.J., Brauns, A.-K., Luers, G., Abrantes, J., Esteves, P.J., da Cruz e Silva, O.A.B., *et al.* TCTEX1D4, a novel protein phosphatase 1 interactor: connecting the phosphatase to the microtubule network. Biology Open. 2013.

71. Zhang, Q., Rubenstein, J.N., Jang, T.L., Pins, M., Javonovic, B., Yang, X., Kim, S.J., Park, I., Lee, C. Insensitivity to transforming growth factor-beta results from promoter methylation of cognate receptors in human prostate cancer cells (LNCaP). Mol Endocrinol. 2005;19(9):2390-9. Epub 2005/05/21.

72. Blanchere, M., Mestayer, C., Saunier, E., Broshuis, M., Mowszowicz, I. Transforming growth factor beta in the human prostate: Its role in stromal-epithelial interactions in non-cancerous cell culture. The Prostate. 2001;46(4):311-8.

73. Walker, L., Millena, A.C., Strong, N., Khan, S.A. Expression of TGFbeta3 and its effects on migratory and invasive behavior of prostate cancer cells: involvement of PI3-kinase/AKT signaling pathway. Clinical & experimental metastasis. 2013;30(1):13-23. Epub 2012/06/09.

74. Vo, B.T., Cody, B., Cao, Y., Khan, S.A. Differential role of Sloan-Kettering Institute (Ski) protein in Nodal and transforming growth factor-beta (TGF-beta)-induced Smad signaling in prostate cancer cells. Carcinogenesis. 2012;33(11):2054-64. Epub 2012/07/31.

75. Czekanska, E.M. Assessment of cell proliferation with resazurin-based fluorescent dye. Methods Mol Biol. 2011;740:27-32. Epub 2011/04/07.

76. Dienstknecht, T., Ehehalt, K., Jenei-Lanzl, Z., Zellner, J., Muller, M., Berner, A., Nerlich, M., Angele, P. Resazurin dye as a reliable tool for determination of cell number and viability in mesenchymal stem cell culture. Bulletin of experimental biology and medicine. 2010;150(1):157-9. Epub 2010/12/17.

77. Guo, Y.P., Kyprianou, N. Overexpression of transforming growth factor (TGF) beta 1 type II receptor restores TGF-beta 1 sensitivity and signaling in human prostate cancer cells. Cell Growth Differ. 1998;9(2):185-93.

78. Kim, I.Y., Zelner, D.J., Sensibar, J.A., Ahn, H.J., Park, L., Kim, J.H., Lee, C. Modulation of sensitivity to transforming growth factor-beta 1 (TGF-beta 1) and the level of type II TGF-beta receptor in LNCaP cells by dihydrotestosterone. Experimental cell research. 1996;222(1):103-10. Epub 1996/01/10.

79. Nakamura, T., Miller, D., Ruoslahti, E., Border, W.A. Production of Extracellular-Matrix by Glomerular Epithelial-Cells Is Regulated by Transforming Growth Factor-Beta-1. Kidney Int. 1992;41(5):1213-21.

80. Fuxe, J., Karlsson, M.C.I. TGF-beta-induced epithelial-mesenchymal transition: A link between cancer and inflammation. Seminars in cancer biology. 2012;22(5-6):455-61.

81. Stephens, F.O., Aigner, K.R. Basics of Oncology: Springer-Verlag Berlin Heidelberg; 2009.

APPENDIX

8.1 PCMV-MYC VECTOR

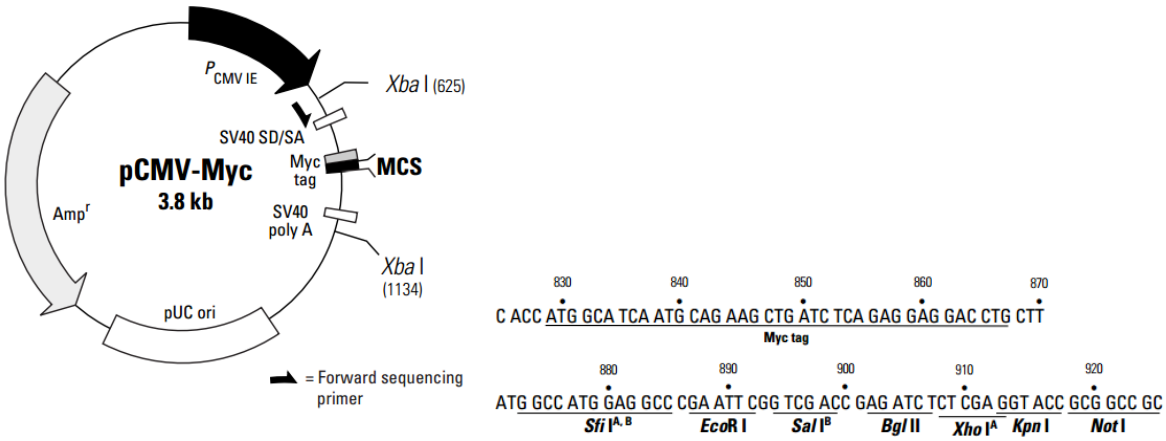


Figure 18 | Restriction map and multiple cloning site (MCS) of pCMV-Myc.

The pCMV-Myc Mammalian Expression Vector (Clontech, Saint Germain-en-Laye, France) expresses proteins containing the N-terminal c-Myc epitope tag. The c-Myc epitope tag is well-characterized and highly immunoreactive. High-level expression in mammalian cells is driven from the human cytomegalovirus immediate early promoter/enhancer (PCMV IE). The vector contains an intron (splice donor/splice acceptor); the epitope tag; an MCS; and a polyadenylation signal from SV40.This vector also possesses the ampicillin resistance gene for selection in *E. coli*.

8.2 ANTIBODIES

Table 3 | Antibodies used for both Western blotting and immunocytochemistry.
Antibodies' target and specific dilution is also stated**.**

Primary antibody	Target	Dilution	Use	Secondary antibody	Dilution
CBC3C	PPP1CC	1:5000	Western blotting	Infrared IRDye-labeled anti-rabbit	1:5000
CBC8C	TCTEX1D4	1:50	Western blotting	Infrared IRDye-labeled anti-rabbit	1:50
Mouse anti-Myc-tag	Myc-tag	1:5000	Western blotting	Infrared IRDye-labeled anti-mouse	1:5000
c-Myc	Myc-tag	1:5000	Immuno-cytochemistry	Texas Red goat anti-mouse	1:300

8.3 BCA PROTEIN ASSAY STANDARDS

Table 4 | Standards used in the BCA protein assay method.
BSA, bovine serum albumin solution (2 mg/ml); SDS, sodium dodecyl sulfate; WR, working reagent.

Standard	BSA (µl)	1% SDS (µl)	Protein mass (µg)	WR (µl)
P0	-	25	0	200
P1	0.5	24.5	1	200
P2	1	24	2	200
P3	2.5	22.5	5	200
P4	5	20	10	200
P5	10	15	20	200

8.4 CELL CULTURE MEDIA AND SOLUTIONS

PBS (1X)

For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Thermo Scientific, Bremen, Germany) in deionized H_2O. Final composition:

- 8 mM Sodium Phosphate
- 2 mM Potassium Phosphate
- 140 mM Sodium Chloride
- 10 mM Potassium Chloride

Sterilize by filtering through a 0.2 μm filter and store at 4°C.

Complete RPMI-1640 Medium

▪ RPMI-1640 (Gibco, Madrid, Spain)	7,945 g
▪ $NaHCO_3$ (Sigma-Aldrich, Sintra, Portugal)	0.425 g
▪ 1% Streptomycin/Penicillin/Amphotericin solution (Gibco, Madrid, Spain)	10 ml
▪ 10% FBS (Gibco, Madrid, Spain)	100 ml

Dissolve RPMI-1640 and $NaHCO_3$ in deionized H_2O. Adjust the pH to 7.2-7.3. Adjust the volume to 500 ml with deionized H_2O. Add Streptomycin/Penicillin/Amphotericin solution and FBS. Sterilize by filtering through a 0.2 μm filter and store at 4°C

Complete Keratinocyte-Serum Free Medium (K-SFM)

▪ K-SFM (Gibco, Madrid, Spain)	500 ml
▪ BPE (Gibco, Madrid, Spain)	25 mg
▪ EGF (Gibco, Madrid, Spain)	2.5 μg
▪ 1% Streptomycin/Penicillin/Amphotericin solution (Gibco, Madrid, Spain)	10 ml
▪ 10% FBS (Gibco, Madrid, Spain)	100 ml

For a final volume of 500 ml of K-SFM (Gibco, Madrid, Spain) add 0,05 mg/ml BPE (Gibco, Madrid, Spain) and 5 ng/ml EGF (Gibco, Madrid, Spain). Add 1% Streptomycin/Penicillin/Amphotericin solution and 10% FBS. Sterilize by filtering through a 0.2 μm filter and store at 4°C.

1 mg/ml poly-L-ornithine solution (10X)

For a final volume of 100 ml, dissolve in deionized H_2O 100 mg of poly-L-ornithine (Sigma-Aldrich, Sintra, Portugal). Sterilize by filtering through a 0.2 μm filter and store at 4°C.

TGFβ1 solution

TGFβ1 (Sigma-Aldrich, Sintra, Portugal) was reconstituted using 0.2 μm filtered 4 mM HCl (Sigma-Aldrich, Sintra, Portugal) containing 1 mg/ml of BSA (Nzytech, Lisboa, Portugal). Stock solution (1 μg/ml) was stored at -20°C.

8.5 IMMUNOFLUORESCENCE SOLUTIONS

4% paraformaldehyde fixative solution

For a final volume of 100 ml, add 4g of paraformaldehyde (Sigma-Aldrich, Sintra, Portugal) to 25 ml deionized H_2O. Dissolve by heating the mixture at 58ºC while stirring. Add 1-2 drops of 1 M NaOH to clarify the solution and filter (with a 0.2 μm filter). Add 50 ml of 2X PBS and adjust the volume to 100 ml with deionized H_2O.

Permeabilization solution

- 0.2% (v/v) Triton X-100 (Sigma-Aldrich, Sintra, Portugal)
- 1X PBS

8.6 SDS-PAGE AND IMMUNOBLOTTING SOLUTIONS

4X LGB (lower gel buffer)

To 900 ml of deionized H_2O add:

- 1.5 M Tris-HCl 181.65 g
- 4% SDS 4 g

Mix until the solutes have dissolved. Adjust the pH to 8.9 and adjust the volume to 1L with deionized H_2O.

5X UGB (upper gel buffer)

To 900 ml of deionized H_2O add:

- 0.6 M Tris-HCl 75.69 g

Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1L with deionized H_2O.

30% Acrylamide/0.8% Bisacrylamide

To 70 ml of deionized H_2O add:

- Acrilamide 29.2 g
- Bisacrylamide 0.8 g

Mix until the solutes have dissolved. Adjust the volume to 100 ml with deionized H_2O. Filter through a 0.2 μm filter and store at 4 °C.

10% APS (ammonium persulfate)

In 10 ml of deionized H_2O dissolve 1 g of APS.

10% SDS (sodium dodecyl sulfate)

In 10 ml of deionized H_2O dissolve 1 g of SDS.

(4X) Loading gel buffer

- 250 mM Tris-HCl solution (pH 6.8) 2.5 ml
- 8% SDS 0.8 g
- 40% Glycerol 4 ml
- 2% β-Mercaptoethanol 2 ml
- 0.01% Bromophenol blue 1 mg

Adjust the volume to 10 ml with deionized H_2O. Store in darkness at RT.

1 M Tris (pH 6.8) solution

To 150 ml of deionized H_2O add:

- Tris base 30.3 g

Adjust the pH to 6.8 and adjust the final volume to 250 ml.

10X Running Bbuffer

- 250 mM Tris-HCl 30.3 g
- 2.5 M Glycine 144.2 g
- 1% SDS 10 g

Dissolve in deionized H_2O, adjust the pH to 8.3 and adjust the volume to 1 L.

10X Transfer buffer

- 25 mM Tris-HCl 3.03 g
- 192 mM Glycine 14.41 g

Mix until solutes dissolution. Adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with deionized H_2O. Just prior to use add 200 ml of methanol (20%).

10X TBS (Tris buffered saline)

- 10 mM Tris-HCl 12.11 g
- 150 mM NaCl 87.66 g

Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionized H_2O.

10X TBST (TBS + Tween)

- 10 mM Tris-HCl 12.11 g
- 150 mM NaCl 87.66 g
- 0.05% Tween 20 5 ml

Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionized H_2O.

Blocking solution

TBST (1X) in 5% of low-fat milk.

Antibody solution

TBST (1X) in 3% of low-fat milk.

8.7 HTS TRANSWELL PERMEABLE SUPPORT

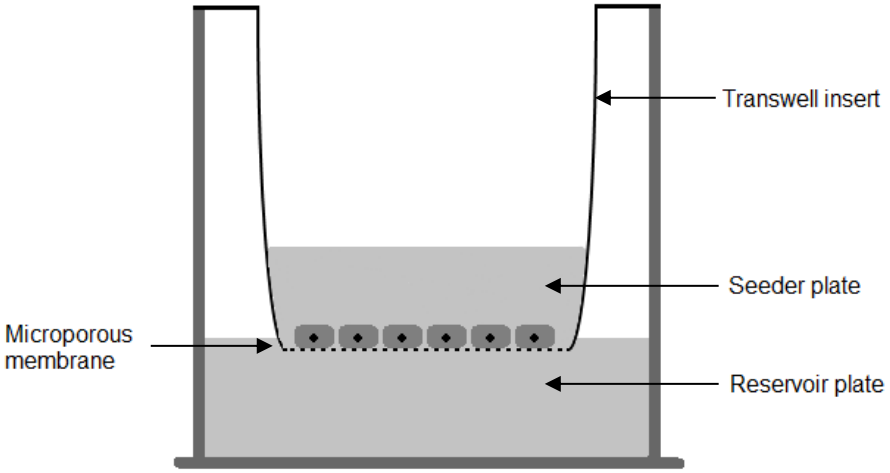


Figure 19 | Scheme of Corning Transwell Permeable Supports.

(VWR International, Carnaxide, Portugal).

This system is constituted by an upper compartment (seeder plate) where cells are seeding, a lower compartment (reservoir plate) which can be accessed via windows in the insert wall, and a microporous membrane through which cells are able to migrate. Different membrane types, pore sizes, and pore density are available.